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# STUDIES IN THE METABOLISM OF PLANT CELLS

## V. SALT RESPIRATION AND ACCUMULATION IN RED BEET TISSUE

by R. N. ROBERTSON[1], J. S. TURNER[2] AND M. J. WILKINS

(From the Botany School, University of Sydney).

*(Accepted for publication 21st October, 1946.)*

In a recent publication, Bennet-Clark and Bexon (1943) have described investigations on beet tissue which considerably increase our knowledge of the respiratory metabolism of plant cells. Somewhat similar work is in progress here on carrot tissue and it has become clear that in both tissues the respiration reacts in somewhat the same way, for example to certain organic acids. We also know a good deal about the uptake of ions by carrot tissue and its relation to respiration, and it seems useful to enquire whether carrot and beet tissue are similar in this respect. The data of Bennet-Clark and Bexon concerning respiratory drift with time and with salt solutions suggest that the two tissues are different in some ways. The methods they used for beet also differed somewhat from ours and we have therefore investigated beet tissue, using precisely the methods that we have developed for carrot tissue.

### MATERIAL AND METHODS.

Red beetroot, unknown variety, was purchased in the dormant stage and slices of 0·8 cm. diam. and 0·25, 0·5 and 1 mm. thick were used for experiment. The roots were well washed and peeled thickly. For some experiments cylinders were cut vertically from the roots with a cork borer and cut by hand into discs 1 mm. thick. For the other experiments, cylinders were cut into a block of beet and left in the block. This was then clamped into a microtome and discs either 1·0, 0·5 or 0·25 mm. in thickness were cut from it. All discs were immediately rinsed several times in distilled water and then aerated in distilled water until required. Slices were used at periods varying from 1–640 hours after cutting.

Respiration rate was determined at 25° C. by measuring the oxygen uptake of 1·5 gm. to 2·0 gm. of tissue in Warburg vessels shaken at 100 oscillations per minute. Tissue to be used was lightly blotted dry, weighed and transferred to vessels with distilled water; the volume of water in the different experiments was varied when different amounts of tissue were used so that the tissue-solution volume ratio remained practically constant. After a steady respiratory drift was reached in distilled water, potassium chloride solution was added from the side arm. The concentration of the potassium chloride added was sufficient to give a concentration of 0·01 M, 0·02 M or 0·04 M, as required, in contact with the tissue. In controls the same volume of distilled water was added from the side arm. The potassium cyanide was added, when desired, by stopping the experiment temporarily, removing the vessels from the manometers and adding the required amount of potassium cyanide to the solution in contact with the tissue: the potassium hydroxide in the centre tube of the vessel was replaced with potassium hydroxide containing the appropriate quantity of potassium cyanide to give a vapour pressure of hydrogen cyanide in equilibrium with that in the solution (Krebs, 1935).

Salt accumulation was measured with replicate sets of tissue held in flasks in the thermostat and attached to the Warburg shaker to ensure aeration. A greater volume of tissue (5–8 gm.) was used in the replicates than in the Warburg vessels but the same tissue-solution volume ratio was maintained. Accumulation was followed by withdrawing samples of the external solution in conductivity pipettes and measuring the changes in conductivity. The samples were then returned to the vessels thus avoiding loss of solution. Advantages of the conductivity method have been discussed in a previous paper in this series.

Respiration results are expressed as mm.$^3$ $O_2$/hr./gm. fresh weight and accumulation results as gm. mol. accumulated/hr./gm. fresh weight.

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[1]Now of Division of Food Preservation, C.S.I.R.

[2]From the Botany School, University of Melbourne.

## RESULTS.

### 1. RESPIRATION.

*Time-drift in Distilled Water.*

It has become apparent from work on carrot tissue (Turner, 1940) that respiratory metabolism is radically altered when a tissue is cut into slices. Experiments with freshly cut slices will not yield the same results as those carried out with slices washed for several days. In carrot root tissue, this change in metabolism is associated with an initial sharp rise and a subsequent continual fall in the rate of respiration. A typical rate-time curve is plotted in Fig. 1C.

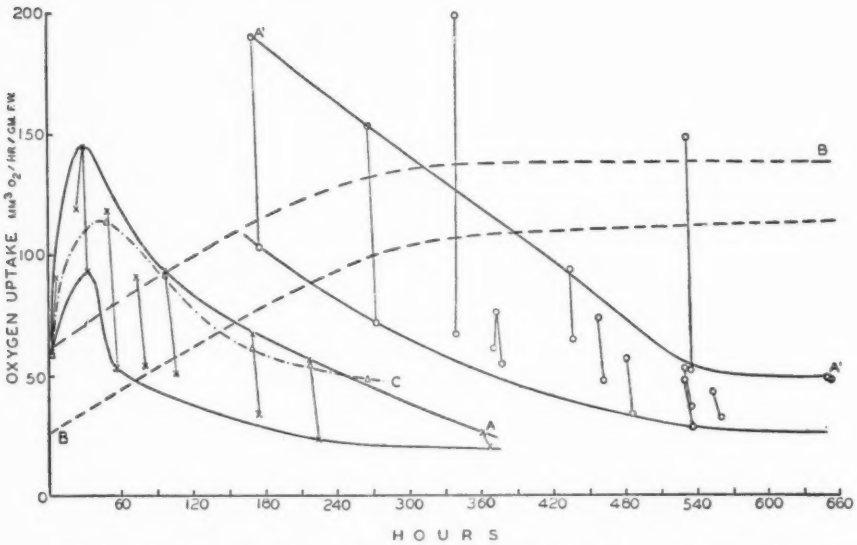


**Fig. 1.** Drift in respiration rates, (in water) with time from cutting: curve A, *beet*, time from cutting experiments; curve A′, *beet*, results collected from all other experiments. The initial and final respiration rates in each experiment are plotted and joined to show the magnitude of the change during the Warburg experiment. Curve B (*beet*) is from the paper of Bennet-Clark and Bexon. Curve C is a typical time drift for cut dormant *carrot* tissue.

For beet tissue, on the other hand, Bennet-Clark and Bexon (1943) report that the "respiratory activity rises fairly rapidly with aging (*i.e.* time from cutting) and attains a value of about four times the initial rate at about 300 hours;" between 300 and 600 hours the rate remains steady at this high value (Fig. 1B). The authors point out that the beet tissue resembles potato more closely than carrot in respect of wounding, although they could find no incipient cambial activity in the beet slices.

Our beetroot slices were all cut from dormant roots and in respect of the time-drift of respiratory activity they closely resembled carrot slices. In Fig. 1A we plot the results obtained from a series of experiments specially planned to determine the time-drift over 360 hours. The respiration rate rose to its maximum (about 2½ times the initial rate) within 30 hours of cutting and then fell, at first rapidly, then more slowly to a value which was 17 p.c. of the maximum and less than 50 p.c. of the initial rate. On the same Figure (curve A′) we plot results assembled from the water-controls of all our other experiments. These were made with roots of a different origin from those used for 1A, and the experiments were spread over several months. In general they confirm the form of the respiration-time curve shown by 1A, although the early values are not given and the average rates are higher.

In most of our experiments the rate of respiration also fell during the short period of the Warburg experiments (usually six hours). In Fig. 1 this fall during an experiment is shown by the straight lines joining each pair of points in both curves A and A′. The fall is steeper than that usually recorded for carrot tissue and, as will be seen from Figs. 2 and 3, it is not linear. This is in contrast to the results of Bennet-Clark and Bexon, whose graphs show steady

or rising rates of respiration during each short experiment. We found rising rates only in tissue cut for less than 30 hours, during which time the "wound respiration" which follows cutting is rising to its maximum value. (Fig. 1A).

*Respiration in Potassium Chloride Solution.*

In almost all our experiments the respiration rate of beet tissue increased rapidly when the water surrounding the tissue was replaced by dilute solutions of potassium chloride. Only when the tissue is freshly cut is this salt effect negligible. In Fig. 2 are plotted the results of an experiment in which a typical salt effect was recorded. The increase was 33 mm.$^3$ $O_2$/hr./gm. F.W., when the rate in water was 65 mm.$^3$ $O_2$/hr./gm. F.W. The form of the curves is very similar to that for carrot (Robertson and Turner, 1944) and the half-time for the effect to reach its maximum is 15–30 minutes. Once the maximum rate in salt is reached, the total respiration rate drifts downwards, usually rather more rapidly than does that of the control in water, so that the ratio "total respiration rate/rate in water" remains roughly constant. As in earlier work, the difference between the rate of "total respiration" (in salt) and that in distilled water will be termed the rate of salt respiration. Results for typical experiments are set out in Table 1.

TABLE 1.

Oxygen uptake ($R$ — mm.$^3$ $O_2$/hr./gm. fresh weight). All values for respiration in water taken just *prior* to the addition of salt; all values for salt respiration and total respiration taken 1½ hours *after** the addition of salt.

| Experiment. | Disc thickness (mm.) | Concentration of potassium chloride (Molar) | Time from cutting. (hrs.) | Respiration in water. $R(w)$ | Salt respiration. $R(s)$ | Total respiration. $R(t)$ |
|---|---|---|---|---|---|---|
| a. | 0·25 | 0·04 | 200 | 64 | 44 | 104 |
| | | 0·02 | approx. | 64 | 30 | 92 |
| | | 0·01 | | 64 | 25 | 87 |
| | | 0·01 | | 72 | 28 | 97 |
| b. | 1 | 0·04 | 528 | 47 | 17 | 57 |
| | | 0·02 | | 45 | 18 | 53 |
| c. | 1 | 0·02 | 528 | 42 | 28 | 66 |
| | | 0·01 | | 39 | 21 | 56 |
| d. | 0·25 | 0·02 | 336 | 78 | 23 | 92 |
| | 0·5 | | | 88 | 28 | 104 |
| | 1·0 | | | 90 | 22 | 100 |

*Hence $R(t)$ is not identical with $R(w) + R(s)$.

*Salt concentration.* The salt effect varies with the time from cutting, but in general, for slices of the same age, salt concentrations of 0·01 M are sufficient to cause the maximal effect. In some experiments, like that depicted in Fig. 2, the salt concentration had to be raised to 0·04 M before maximal salt respiration was induced. In respect of salt effect there is little difference between beet and carrot tissue; in both, the limiting salt concentrations are usually below 0·01 M, the salt effect varies a good deal with the age and source of the tissue and the time relations are approximately the same.

*Slice thickness.* The maximum $QO_2$ ($\mu$l. $O_2$/mg. D.W./hr.) for beet is 5·6; for carrot it is 3·6. One would therefore expect tissue slices of 1 mm. thickness to provide suitable material for Warburg experiments (*vide* Turner, 1938). In preliminary experiments slices of three different thicknesses were cut consecutively from beet roots (to give random sampling) and the respiration rates of paired samples 0·25 mm., 0·5 mm. and 1 mm. thick are recorded in Fig. 3. When the respiration-time curves drifting downwards had been established, potassium chloride (0·02 M) was added to one sample of each pair, and the salt effect was measured. All the slices had been washed for 336 hours.

The thinner the slice, the greater is the ratio of damaged surface cells to undamaged internal cells; when allowance is made for this there is no consistent significant difference in the respiration rates of thin and thick slices, (over the range 0·25–1·0 mm.) and the salt effect is the same for all three thicknesses of slice.

*The Aging Effect.* Recent work with carrot, (to be published shortly) has shown that the salt effect is almost negligible with freshly cut tissue slices and becomes pronounced only after the respiration rate has passed its peak. The same appears to be so for beet tissue, although no very extensive experiments have been carried out. In Fig. 4 we plot respiration in water and the comparable total respiration in salt solution. In the series of experiments, 4A, the salt effect increases gradually during the initial rise and fall of the respiration in water, and after some

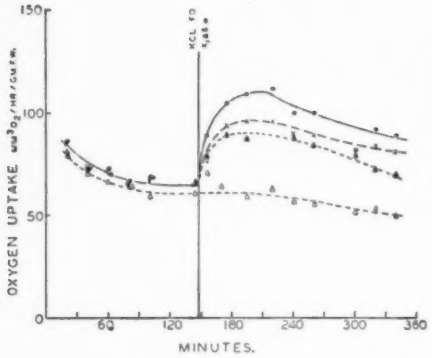


**Fig. 2.** Respiration rates (*beet*) plotted against time, with different concentrations of potassium chloride.

Open triangles, control, in water. Solid triangles, tissue receiving 0·01 M. KCl; Crosses, tissue receiving 0·02 M. KCl; Solid circles, tissue receiving 0·04 M. KCl; Salts added at 148 minutes.

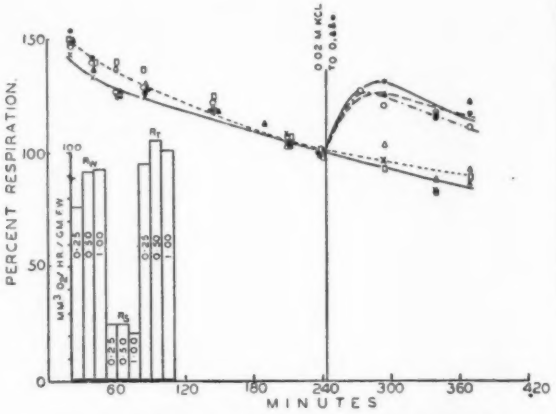


**Fig. 3.** Respiration rates for (*beet*) discs of different thickness, plotted against time; the rates for each set are plotted as percentages of that immediately prior to the addition of the salt.

Cross and open circle, discs 1 mm. thick; Open and solid triangle, discs 0·5 mm. thick; Square and solid circle, discs 0·25 mm. thick.

The histograms (inset) give a comparison of the absolute rates. *Rw*, rate in water just prior to addition of salt; *Rs*, salt respiration and *Rt*, total respiration at the end of the experiment.

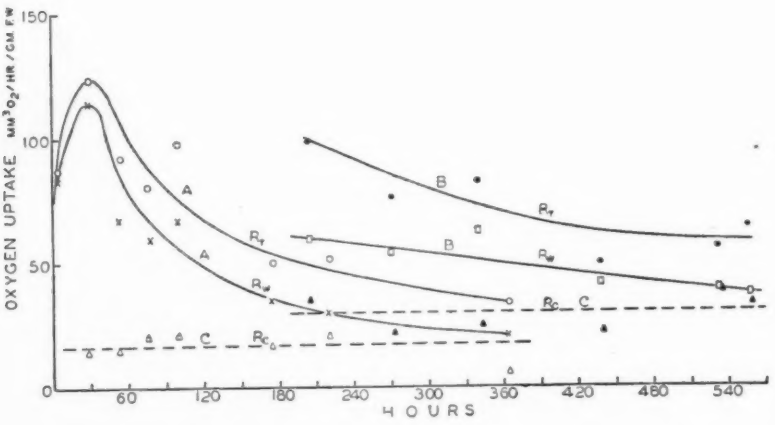


**Fig. 4.** Respiration rates (*beet*) in water (*Rw*), in salt solution (*Rt*) and in cyanide (*Rc*), plotted against time of aging in distilled water. Each point represents the mean steady rate obtained from one Warburg experiment.

200 hours the salt respiration equals more than 50 p.c. of the respiration in water. In series 2B, the rate in water is higher than in series 2A and the salt effect varies little between 260 and 560 hours. For our present purpose—comparison of beet and carrot tissue—it is sufficient to point out that there is a well marked salt effect in beet at all periods except immediately after cutting. To some extent Bennet-Clark and Bexon confirm this, but they are of the opinion that potassium chloride at 0·05 M brings about an increase in rate of only 1 p.c. per hour—stating "these salt

effects are hardly appreciable during the short experimental period of 2–6 hours." On the contrary we find marked salt effects, sometimes doubling the respiration rate in less than one hour. We shall return to this discrepancy in the discussion.

*Sensitivity of the Respiration to Cyanide.*

In the majority of our experiments cyanide reduces the rate of both salt respiration and respiration in water; for comparable tissue samples, cyanide reduces the total respiration and the water-respiration to the same level. As in carrot tissue, a cyanide concentration of M/1,000 is sufficient to inhibit all the cyanide-sensitive respiration.

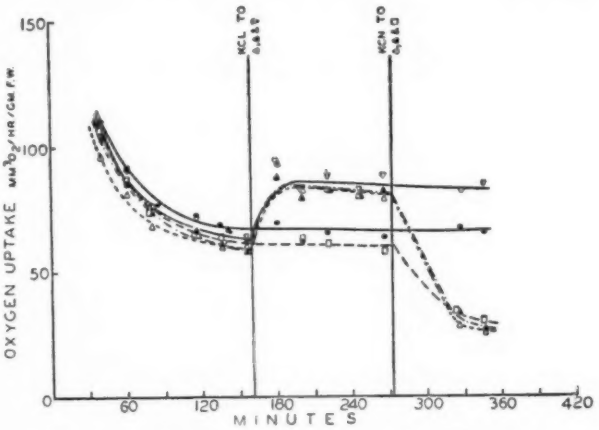


**Fig. 5.** Respiration in water, salt solution (KCl) and potassium cyanide solution, plotted against time. (*Beet*).

Solid circles, control in water; Squares, tissue receiving 0·001 M. KCN; Inverted triangle, tissue receiving 0·02 M. KCl; Triangle, tissue receiving 0·02 M. KCl and later 0·001 M KCN; Solid triangle, tissue receiving 0·02 M. KCl and later 0·01 M. KCN.

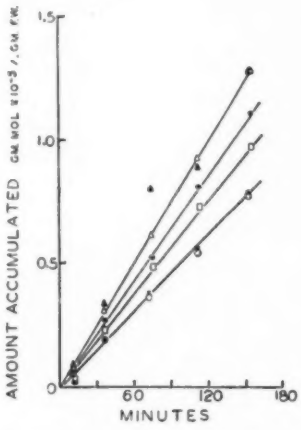


**Fig. 6.** Amount of potassium chloride accumulated by beet discs of different thickness, plotted against time.

Cross and open circles, discs of 0·25 mm. thickness; Open and solid triangles, discs of 0·5 mm. thickness; Squares and solid circles, discs of 1 mm. thickness.

In only two experiments, in both of which the tissue had been washed for many hours, was the "water-respiration" unaffected by cyanide; in carrot tissue also, the water respiration may be partially sensitive to cyanide for periods up to 300 hours from cutting. For all our experiments with beet it is possible to summarize the results by stating that the "total" respiration was reduced by cyanide to the level of the control in water to which the same amount of cyanide had been added. For example, we plot in Fig. 5 a typical experiment for which the slices had been washed for 336 hours and after which the respiration was measured for 160 minutes in water. By this time the respiration rates were steady and 0·02 M KCl was added to 3 of the 5 sets. After a further 110 minutes, during which well marked salt effects were produced, cyanide at two concentrations (0·01 M and 0·001 M) was added to three of the sets. The respiration rates fell in the presence of cyanide to approximately the same value, whether or not chloride had been previously added, and this value was well below that of the control in water.

Salt respiration, then, in beet tissue is completely sensitive to cyanide, but so also is a part of the respiration in distilled water. The cyanide-insensitive fraction of beet respiration remains roughly constant however long the slices are washed. It is obvious from Figure 4 that the early high "wound respiration," like the salt respiration, is sensitive to cyanide and is completely inhibited by 0·001 M KCN.

### 2. SALT ACCUMULATION.

With each respiration experiment, uptake of ions from the external solution was studied in replicate sets of tissue. Immediately after the addition of potassium chloride there was a rapid uptake of salt over a period of 20–30 minutes. After this initial rapid uptake,

*accumulation* proceeded at a steady rate for at least three hours. In some experiments accumulation rates began to decrease after that time. Fig. 6 shows the results of a typical experiment, salt accumulation being plotted as gm. mol. accumulated/gm. F.W. The slices had been washed for 264 hours.

*Salt Concentration.*

The rate of accumulation approached an asymptotic value with increasing external concentration of potassium chloride. This has also been observed with carrot tissue. For beet the concentration at which the apparent maximum accumulation rate is reached varies in different batches of tissue, as may be seen in Table 2.

TABLE 2.

*Accumulation rate—gm. mol. accumulated/hr./gm. fresh weight.*

| Experiment. | Conc. KCl. Molar. | Accumulation Rate. |
|---|---|---|
| 1 | 0·005 | $0{\cdot}11 \times 10^{-5}$ |
| | 0·01 | $0{\cdot}24 \times 10^{-5}$ |
| | 0·02 | $0{\cdot}50 \times 10^{-5}$ |
| | 0·02 | $0{\cdot}30 \times 10^{-5}$ |
| 2 | 0·01 | $0{\cdot}24 \times 10^{-5}$ |
| | 0·01 | $0{\cdot}20 \times 10^{-5}$ |
| | 0·02 | $0{\cdot}26 \times 10^{-5}$ |
| | 0·02 | $0{\cdot}24 \times 10^{-5}$ |
| | 0·04 | $0{\cdot}27 \times 10^{-5}$ |
| 3 | 0·01 | $0{\cdot}29 \times 10^{-5}$ |
| | 0·02 | $0{\cdot}35 \times 10^{-5}$ |
| | 0·04 | $0{\cdot}49 \times 10^{-5}$ |

*Disc Thickness.*

In Fig. 6 the effect of slice thickness on accumulation is illustrated. In this Figure the initial rapid uptake is omitted and the subsequent accumulation, for discs of 0·25 mm., 0·5 mm. and 1 mm. thickness, is plotted against time. As with similar respiration experiments, the slices of three different thicknesses were cut consecutively from the roots. Discs of these three thicknesses show similar accumulatory powers per unit of fresh weight. The minor differences in rate may be explained as follows: the proportion of injured (and non-living) cells will be greatest in the thinnest discs; therefore, relative to total weight these discs will contain the fewest cells capable of accumulation. It is possible that in the thickest discs (1 mm.) some cells near the middle will not accumulate as rapidly as those towards the edge of the disc, but there is no evidence that accumulatory activity is restricted to the undamaged surface cells.

*Cyanide Sensitivity of Accumulation.*

In Fig. 7 the changing *conductivity* of the external solution is plotted, to illustrate the typical effect of potassium cyanide on salt accumulation. Two tissue sets received first 0·02 M potassium chloride, two others were given 0·01 M chloride. The accumulation rates, judged by the conductivity of the external solution, were followed for nearly two hours and then one member of each paired set was given 0·001 M potassium cyanide. This caused an immediate increase in conductivity, due to the cyanide, but was followed by a cessation of accumulation. All the discs had been washed for approximately 200 hours.

The other experiment illustrated in this graph showed that if potassium chloride and cyanide are added at the same time to tissue previously in water, an initial rapid uptake (of the chloride) takes place, but there is no subsequent accumulation, the external conductivity remaining unchanged after the first hour. This confirms the view that accumulation and cyanide-sensitive salt respiration are intimately related.

## DISCUSSION.

Our results indicate that beet tissue and carrot tissue are broadly similar with regard to the wound effect and to their power of accumulating ions. In both tissues, cutting dormant roots into thin slices causes the respiration to rise to a maximum within 30–70 hours; the rate subsequently falls, reaching after about 300 hours a low rate which then declines very slowly. This rise and fall in rate is shown by slices of carrot and beet tissue washed throughout in distilled water and it is also shown in carrot tissue washed in tap water poor in dissolved mineral

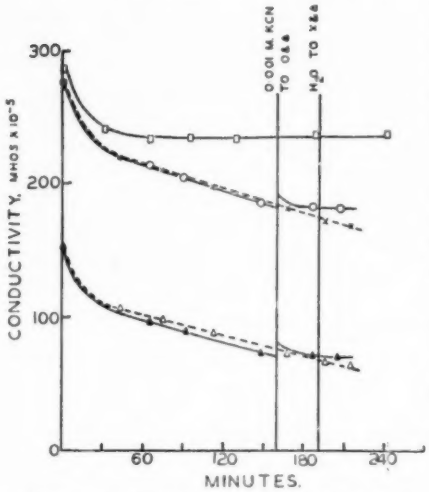


**Fig. 7.** Conductivities of external solutions in contact with beet tissue.

Squares, tissue in 0·02 M. KCl and 0·001 M. KCN; Cross, tissue in 0·02 M. KCl; Triangle, tissue in 0·01 M. KCl; Circle, tissue in 0·02 M. KCl and later receiving 0·001 M. KCN; Solid triangle, tissue in 0·01 M. KCl and later receiving 0·001 M. KCN.

substances (Turner, 1940). In carrot, the final low rate of respiration in the well washed slices is of the same order as that in uncut dormant roots, but we do not yet know whether the same can be said of beet tissue.

When both carrot and beet tissue are placed in dilute potassium chloride solutions within five or six hours of cutting, there is no change in the respiration rate. However, both tissues are closely similar in that, when they have been washed for longer periods in distilled water, transference to dilute chloride solution initiates a salt respiration. The rate of salt respiration is low in these slices, but it increases as the preliminary time of washing is increased until, in tissue washed for 300 hours the salt respiration may be 60 p.c. of the control rate in water (Fig. 4).

The salt effect, in both tissues, is very quickly established, the half time for reaching the maximum being between 15 and 30 minutes. In both beet and carrot tissue a potassium chloride solution of 0·01 M concentration is usually sufficient to cause maximal salt effect.

In both tissues there is also a rapid uptake of potassium choride within 15 minutes of first placing it in contact with the tissue. Following this, both tissues *accumulate* the salt and simultaneously the rate of respiration (oxygen uptake) is increased.

Further, in both tissues, salt respiration is completely inhibited by potassium cyanide at concentrations as low as M/1000. Simultaneously, the accumulation of salt by the poisoned cells is completely stopped, although the salt previously accumulated is not lost from the cells.

For both tissues, when the washing time lies between 12 and 100 hours, cyanide inhibits part of the "wound" respiration in water as well as the whole of the salt respiration. After about 100 hours, well washed carrot tissue in water is often completely insensitive to cyanide, which then only inhibits salt respiration. In similarly "aged" beet tissue used in these experiments the water respiration of the controls remained sensitive to cyanide, *i.e.* in salt-stimulated tissue, cyanide inhibited more than the salt respiration. Thus beet tissue, like the roots with which Lundegårdh (1940) and Machlis (1944) worked, does not provide such a striking differentiation of "salt" and "water", or ground respiration, as does the carrot. Provisionally we suppose that, on cutting both beet and carrot tissue, a cyanide-sensitive wound respiration is superimposed upon a basal cyanide-stable respiration; in carrot, during "aging" the wound respiration usually, though not always, drifts to zero within 100 hours, whereas in our variety of beet it is still active( but at a low rate) even 600 hours after cutting.

We have now to consider how far our results confirm those of Bennet-Clark and Bexon. They report that for their beet tissue, "the most notable feature of the behaviour of the discs is that their respiratory activity rises fairly rapidly on

aging and attains a value about four times the initial rate about 300 hours after cutting" (see Fig. 1). It is difficult to reconcile this observation with our own. It is to be noted that they washed their discs in tap water, not distilled water, so that a salt effect was always present. However, this probably cannot explain the discrepancy that we are discussing, because (a) the tap water used was very poor in mineral content (private communication), (b) the maximal rates reached were very high, (c) washing in tap water does not materially alter the rate-time drift for carrots (Turner, 1940).

Bennet-Clark and Bexon state that "beet itself shows different courses of aging effect dependent on whether the discs are cut during the growing phase, the dormant phase or the sprouting phase." As they also state that it is fully dormant beet that is dealt with in their paper (p. 79), presumably the rate-time drift described above refers to tissue of this kind. It was dormant beet that we used in our work.

It is of interest here to point out that the rate-time drift for carrot tissue is summarized for dormant tissue of both English and Australian horticultural varieties by the curve C of Fig. 1. But very recently we have found that for discs cut from carrots dug very late in the season the rising phase of respiration during aging is prolonged and the mean rates are higher. Even in this tissue, however, the high rates are not prolonged (as in Fig. 1B) and there is a typical, though small salt effect.

Bennet-Clark and Bexon also report that their beet tissue was not sensitive to dilute potassium chloride except over very long periods during which the salt caused an increase in respiration at a rate of only 1 p.c. per hour. Still stronger solutions of potassium chloride are reported to have no effect on the respiration even over a period of five hours, apart from those strengths which cause plasmolysis. Our results are only apparently in conflict with these, as Bennet-Clark and Bexon placed the slices in salt solution immediately after cutting, at a period when the tissue is insensitive to salt.

As a result of this work, it is concluded that research on tissue respiration should take into account the salt effect and, in particular, the change in metabolism which occurs during the preliminary washing of the discs. Further work is required to explain the inconsistencies noted and to establish the typical form of the drift of respiration with time in cut beet tissue.

## SUMMARY.

The effects of dilute potassium choride solution and of potassium cyanide upon the respiration and ion uptake of red beet tissue have been investigated. It is shown that the effects are very similar to those already described for carrot tissue. Beet tissue has the same power of accumulating salt and shows a typical cyanide-sensitive salt respiration. Certain discrepancies between the results and those reported for red beet by Bennet-Clark and Bexon are discussed.

*Acknowledgement.* Our thanks are due to Professor Eric Ashby, in whose department the experiments were done, for his helpful criticism of the manuscript.

## REFERENCES.

Bennet-Clark, T. A. and Bexon, D. (1943): New Phytol., 42, p. 65.
Krebs, H. A. (1935): Biochem. J., 29, p. 1,620.
Lundegårdh, H. (1940): Ann. Agr. Coll. Sweden, 8, p. 234.
Machlis, L. (1944): Amer. J. Bot., 31, 3, p. 183.
Robertson, R. N. (1941): Austral. J. exp. Biol., 19, p. 265.
Robertson, R. N. (1941): *Ibid.*, 22, p. 237.
Robertson, R. N. and Turner, J. S. (1945): *Ibid.*, 23, p. 63.
Turner, J. S. (1938): New Phytol., 37, p. 232.
Turner, J. S. (1940): Austral. J. exp. Biol., 18, p. 273.

# STAPHYLOCOCCAL BACTERIOPHAGES

## I. THE EFFECT OF PENICILLIN ON STAPHYLOCOCCAL BACTERIOPHAGES

by PHYLLIS M. ROUNTREE

(From the Fairfax Institute of Pathology, Royal Prince Alfred Hospital, Sydney).

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Recently, interest has been redirected to the problems presented by the behaviour of staphylococcal bacteriophages. Fisk (1942) described a method for the detection of lysogenic strains of staphylococci and and subsequently Wilson and Atkinson (1945), using this method, developed from lysogenic cultures a series of bacteriophages by means of which a number of phage types of *Staphylococcus aureus* may be identified.

The bacteriolytic action of pencillin on young culture of staphylococci was originally demonstrated by Fleming, and Todd (1945) has drawn attention to some interesting questions connected with this bacteriolytic action. Todd's work suggested that it would be of interest to determine whether lysis by pencillin of lysogenic strains of staphylococci would result in the release of phage from the cells of such strains. It was considered that the result of this action of penicillin on the lysogenic strains might throw some light on the nature of the phage-cell association.

It was reported by Neter and Clark (1944) that penicillin in a concentration of 2,000 units per ml. did not affect the lytic activity of a staphylococcal phage exposed to its action for 18 hours at 37° C. Similarly, Jones (1945) stated that a filtrate of a staphylococcal phage was unaffected by an exposure to 1,000 units of penicillin per ml. for 24 hours at 37° C.

### DESCRIPTION OF STAPHYLOCOCCI AND PHAGES USED.

From a series of 40 strains of *Staphylococcus aureus* obtained from a variety of pathogenic conditions, five lysogenic strains and the appropriate susceptible propagating strains were isolated by the use of Fisk's cross-testing technique. These strains are typical aureus strains, giving a positive coagulase reaction and producing alpha haemolysin and varying amounts of pigment. One strain, S35, obtained from a case of cellulitis treated with penicillin is resistant to a concentration of 50 units of penicillin per ml. The other strains are penicillin sensitive.

The phages from these cultures have been isolated and active filtrates prepared from growth on agar plates. The method of Wilson and Atkinson has been followed in designating these phages by the organism from which they were derived and by the strain on which they are propagated. Table 1 sets out the relationship of the strains.

TABLE 1.

*Relationship of strains of staphylococci and phages.*

| Lysogenic Strain. | Propagating Strain. | Phage Filtrate. |
|---|---|---|
| S14 | S17 | 14/17 |
| S21 | S11 | 21/11 |
| S22 | S25 | 22/25 |
| S33 | S35 | 33/35 |
| S35 | S33 | 35/33 |

It will be noted that S33 and S35 are mutually lysogenic. Both these cultures correspond to Wilson and Atkinson's Type 2A and the phages from them are readily propagated on their Type 2A cultures. S22 corresponds to Wilson and Atkinson's Type 1B; S25 does not clearly fall into any of their types; phage 22/25 does not correspond with any of their phages. Phages 14/17 and 21/11 also are different from Wilson and Atkinson's phages.

The most convenient temperature of incubation was found to be 27° C. All the phages will, however, multiply on agar and in broth at 37° C. but, if agar plates are left at this temperature for longer than 5–6 hours, plaques are small and obscured by the more rapid growth of the staphylococci at this temperature. Although multiplication of the phages can be demonstrated in broth cultures, both at 37° C. and 27° C., complete lysis of the cultures rarely occurs.

All five phages fall into the same serological group. An anti-phage serum prepared in rabbits by immunization with Wilson and Atkinson's phage 51/145 neutralizes to titre all five phages.

There is one point of difference between phages 33/35 and 35/33 and the other three phages used. Lysis of staphylococci on agar plates by phages 33/35 and 35/33 is not inhibited by a concentration of 1·5 p.c. sodium citrate in the medium, while the action of the other three phages is inhibited by a concentration of 0·25 p.c. sodium citrate.

Burnet and Lush (1935), in their study of staphylococcal bacteriophages, found that the aureus phages which they investigated fell into three groups, which they called strong, weak and intermediate. The strong phages produced lysis in broth at 37° C., were not inhibited by 1·5 p.c. sodium citrate and fell into a distinct serological group. These strong phages were readily absorbed by all normal aureus strains, including those on which they did not multiply. The intermediate phage behaved similarly to the strong phage in its absorption on all normal aureus strains; it was, however, serologically distinct, was inhibited by 1 p.c. sodium citrate and could be grown in broth at 37° C. only when a light bacterial inoculum was used. The weak phages could be grown only on agar at 22° C. and on first isolation were inactivated at 37° C. They were inhibited by 0·25 p.c. sodium citrate, formed a distinct serological group and were not absorbed either by killed or living susceptible cultures at refrigerator temperature.

Fisk (1942) found that his phages resembled Burnet and Lush's weak phages in their inability to produce lysis regularly in broth cultures.

An examination of the characteristics of the phages used in this investigation shows that they do not clearly fall into any of the categories defined by Burnet and Lush. By citrate sensitivity tests, they can be divided into citrate sensitive and citrate resistant phages and could, on this basis, be classified as weak and strong. However, they belong to a single serological group and behave similarly to the strong and intermediate phages in their absorption by all normal aureus strains. Further, in their growth temperature behaviour, they resemble intermediate phages.

## METHODS.

The medium used for the propagation of the phages was 1·2 p.c. nutrient agar containing 0·2 p.c. of glucose and adjusted to pH 8·0. Phage counts were made in duplicate by spreading 0·02 ml. quantities on a quarter of an agar plate inoculated with a few drops of a young broth culture of the appropriate organism. Plates were incubated overnight at 27° C. All dilutions of phage, cultures and penicillin were made in broth.

## EXPERIMENTAL.

### *The Effect of Penicillin and Penicillinase on Phage Filtrates.*

As a preliminary step it was thought advisable to repeat the work of Neter and Clark and of Jones on the effect of penicillin on phage filtrates, using the phages isolated in this laboratory. Since it was intended to use penicillinase to neutralize the effect on the propagating staphyloccocci of the penicillin present in inocula used for plaque counts, the action of penicillinase on phage filtrates was also investigated.

TABLE 2.

*Effect of penicillin and penicillinase on phage 14/17.*

| Tube. | Temp. C. | Average Plaque Count. |
|---|---|---|
| Phage and broth | 20° | 85 |
| Phage and broth | 37° | 90 |
| Phage and penicillin | 37° | 93 |
| Phage and penicillinase | 37° | 102 |

Phage filtrates of known titre were diluted in such a manner that inocula from the mixtures of phage and penicillin and of phage and penicillinase would give from 100–200 plaques when plated on agar. Penicillin and penicillinase were diluted to contain a concentration of 50 units per ml. when mixed with the phage. Tubes containing volumes of 1 ml. of phage and 1 ml. of penicillin, 1 ml. of phage and 1 ml. of penicillinase, and 1 ml. of phage and 1 ml. of broth, were incubated at 37° C. for 24 hours. A control tube of phage and broth was also kept at room temperature. At the end of the incubation period, sufficient penicillinase was added to neutralize the effect of the penicillin and plaque counts were made. The results of a typical test, in which phage 14/17 was used, are shown in Table 2.

These results show that neither penicillin nor penicillinase had any effect on the phage filtrate. The variation in the counts is due to technical factors in the spreading of the plates. Similar results were obtained with the other phages, thus confirming the observations of Neter and Clark and of Jones.

*The Effect of Lysis with Penicillin on Lysogenic Staphylococci.*

Tests for bacteriolysis with penicillin were set up with the five lysogenic strains according to Todd's method, 0·9 ml. quantities of a 2½ hours' old broth culture just showing turbidity being mixed with 0·1 ml. of various dilutions of penicillin to give a final concentration of 10, 1 and 0·1 units of penicillin per ml. A control tube of untreated broth culture and a formalinized control for turbidity were included in each series. The tubes were inspected from time to time and, at the end of 8 hours incubation at 37° C., lysis was complete with strains S14, S21, S22 and S33, S35 being resistant, as shown in Table 3.

TABLE 3.

*Bacteriolytic effect of penicillin on staphylococci.*

| | Penicillin concentration/ml. | | | Control | |
|---|---|---|---|---|---|
| Strain. | 10 units. | 1 unit. | 0·1 unit. | Nil. | Formalinized. |
| S14 | ++++ | ++++ | ++++ | 5 | 1 |
| S21 | ++++ | ++++ | ++++ | 5 | 1 |
| S22 | ++++ | ++++ | ++++ | 5 | 1 |
| S33 | ++++ | ++++ | ++++ | 5 | 1 |
| S35 | 5 | 5 | 5 | 5 | 1 |

++++ = complete lysis. 1 = degree of initial turbidity. 5 = degree of final turbidity.

To each of the tubes containing the penicillin-lysed cultures sufficient penicillinase was added for neutralization of the penicillin present—penicillinase was omitted from the control tube containing the actively growing culture. Standard loopfuls were then removed from each tube and spotted on to agar previously spread with a few drops of a young broth culture of the appropriate staphylococcus.

The plates were examined after incubation overnight at 27° C. for evidence of phage action in the form of plaques or rings of inhibition of growth. No evidence of bacteriophage action was found in inocula from any of the tubes containing completely lysed cultures. The actively growing cultures in the control tubes all produced lysis in the form of rings of lysis at the periphery of the loopfuls deposited on top of the susceptible cultures. In the case of the controls from S33 and S35 in addition to the rings of lysis there were also numerous plaques present on the superimposed culture, due to the mutually lysogenic effect of these cultures. The results are summarized in Table 4.

TABLE 4.

*Effect of penicillin lysis on the bacteriophage of lysogenic staphylococci.*

| Penicillin treated strain. | Strain used for plating. | Penicillin concentration/ml. 10 units. | 1 unit. | 0·1 unit. | Nil. |
|---|---|---|---|---|---|
| S14 | S17 | — | — | — | + |
| S21 | S11 | — | — | — | + |
| S22 | S25 | — | — | — | + |
| S33 | S35 | — | — | — | + |
| S35 | S33 | + | + | + | + |

+ = evidence of phage action on plate. — = No evidence.

These results indicate that lysis of the strains tested does not release into the surrounding medium the phages which they carry.

*Phage Absorption by Penicillin Treated Staphylococci.*

Jones (1945) reports some observations on the effect of antibiotic substances including penicillin upon staphylococci and a staphylococcus bacteriophage. Penicillin at a concentration of 10 units per ml. was allowed to act on a susceptible staphylococcus for three hours and then phage was added to the mixture. Counts of the number of viable staphylococci and of the number of phage particles were made at various intervals. The initial phage concentration was $75 \times 10^5$ particles per ml. and this was added to a suspension containing $9 \times 10^3$ viable cells per ml. (the count having been reduced from $114 \times 10^5$ cells per ml. by 3 hours' exposure to penicillin). At the end of a further 5 hours the phage count was $19 \times 10^5$ particles per ml.

On the basis of these results, Jones suggests that penicillin affects the staphylococcal cell in such a manner that it no longer absorbs phage particles. The figures given may, however, be interpreted in a different way when the behaviour of the staphylococcal phages in their absorption on to staphylococci is considered. Phage added to either living or heat-killed susceptible cells is very rapidly absorbed and, in the case of living cells, the first release of phage particles by lysis of the cells occurs within a time which is characteristic for the particular phage but which is usually within an hour. This has been demonstrated by Burnet (1929) using salmonella and staphylococcal phages and by Delbrück (1945) with coli phages. In the case of killed cells no release of absorbed phage particles has been demonstrated. There is a relationship between the density of a bacterial suspension and the amount of phage it will absorb. For example, a killed 18-hour broth culture of S33 diluted 1 in 20 will absorb, within an hour, all the particles in a filtrate containing 20,000 phage particles per ml., whereas the same culture diluted 1 in 200 will absorb only 90 p.c. of the added phage. The counts obtained by Jones 5 hours after the addition of phage to the penicillin treated cells may be due to the effect of adding phage to a mixture of a large number of killed cells and a relatively small number of viable cells.

This question of the absorption of phage on to penicillin treated cells was therefore investigated, making use of the fact that strain S35 is penicillin resistant and lysable by phage 33/35, so that if this phage is used for absorption tests, the need to add penicillinase before making plaque count is obviated. Further, previous experience had shown that strains S17 and S33, although not lysed by this phage, readily absorb and inactivate it. Young broth cultures of S17, S33 and S35, which showed just detectable turbidity, received a dosage of penicillin sufficient to give a concentration of 5 units per ml. and were incubated at 37° C. Untreated broth cultures were also included as controls in the test. At the end of 3 hours' incubation, the penicillin treated cultures of S17 and S33 showed no increase in turbidity, whereas the cultures of the penicillin resistant S35 and the untreated control cultures of S17 and S33 had obviously multiplied during the period. Phage 35/33 was added to each tube in an amount sufficient to give a concentration of 10,000 phage particles per ml. Because the lytic effect of S35 on S33 interfered with reading of the plaque counts, it was found necessary to heat the S33 control culture for 5 minutes in a boiling water bath before the addition of the phage; the S17 control culture was, however, used in the living state. After one hour's absorption at 37° C., plaque counts were made by plating on S35 in the usual way.

TABLE 5.

*Absorption of phage 33/35 by penicillin treated staphylococci.*

| Tube. | Plaque count. | |
|---|---|---|
| S17 + phage | 0 | 0 |
| S17 + penicillin + phage | 5 | 1 |
| S33 + phage | 0 | 1 |
| S33 + penicillin + phage | 0 | 5 |
| S35 + phage | +++ | +++ |
| S35 + penicillin + phage | +++ | +++ |
| Penicillin + phage | 210 | 198 |
| Broth + phage | 224 | 218 |

Table 5 sets out the plaque counts obtained. It will be noted that the phage multiplied on S35 after an hour's contact so much that, on plating, it was impossible to count the number of plaques present on the plates, which showed semi-confluent lysis. There was almost complete absorption of the phage by the penicillin treated cultures of S17 and S33, and by the control cultures of these two strains. The few particles remaining viable in the penicillin treated cultures may be attributed to the lesser concentration of organisms in these cultures.

The tubes were left in the incubator at 37° C. overnight and, on inspection in the morning, S17 and S33 containing penicillin were found to be completely clear, penicillin lysis having occurred. Counts made from these tubes revealed no plaques indicating that the absorbed phage particles are not released on lysis of the cells with penicillin.

These lysed cultures were then treated with an additional dose of phage 33/35, incubated at 37° C. for 1 hour and then examined for evidence of phage absorption by plating on S35. No evidence of phage absorption was found; the results of the plaque counts are given in Table 6.

TABLE 6.

*Effect of penicillin lysis on phage absorption.*

| Penicillin lysed Culture + Phage. | Plaque count. | |
|---|---|---|
| S17 | 70 | 86 |
| S33 | 64 | 79 |
| Phage + Broth | 86 | 80 |

From the above experiments, it is concluded that, contrary to the experience of Jones, staphylococcal cultures exposed to the action of penicillin for 3 hours but with their cells intact will absorb phage in the same way as untreated cultures. When the cells are finally lysed as the result of the penicillin action, the cultures no longer absorb or inactivate phage.

An objection can be made to the applicability of this conclusion in that the results reported concern what has been termed the non-specific resistance of normal staphylococci when acted on by phages which do not lyse them, whereas Jones was dealing with a staphylococcus and its homologous phage. To meet this objection, the experiment has been performed using phage 14/17 and its susceptible strain S17. In this case, a 4-hour-old glucose broth culture of S17 was acted on by a concentration of 5 units of penicillin per ml. at 37° C. for one hour. An untreated culture was included as a control. At the end of an hour, an equal volume of phage 14/17 was added and phage counts were made at various intervals after further incubation at 37° C. It was necessary to add penicillinase before performing the phage counts, since S17, on which the counts were made, is sensitive to penicillin. One drop of undiluted penicillinase was mixed with 0·5 ml. of the phage-penicillin-culture mixture and allowed to act for 5 minutes before plating. The counts obtained 30, 60, 90 and 120 minutes and 21 hours after the addition of the phage are shown in Table 7.

TABLE 7.

*Absorption of phage 14/17 by penicillin treated staphylococcus S17.*

| Time. | Tube. | Plaque count. | |
|---|---|---|---|
| 30 mins. | Phage + culture + penicillin | 26 | 18 |
| | Phage + culture | 386 | 394 |
| | Phage + broth | 556 | 521 |
| 60 mins. | Phage + culture + penicillin | 7 | 7 |
| | Phage + culture | 303 | 298 |
| 90 mins. | Phage + culture + penicillin | 6 | 10 |
| | Phage + culture | 416 | 400 |
| 120 mins. | Phage + culture + penicillin | 20·23 | 10·16 |
| | Phage + culture | +++ | +++ |
| 21 hours | Phage + culture + penicillin | 0 | 0 |
| | Phage + culture | +++ | +++ |

The penicillin-treated cells of S17 inactivated 96 p.c. of the added phage in half-an-hour and at the end of an hour 99 p.c. inactivation had occurred. The 28 p.c. reduction in the count in the phage plus untreated culture tube may be due to the absorption of more than one phage particle on to a proportion of the staphylococcal cells in the culture, such cells on plating only producing one plaque. It will also be noted that the first liberation of phage in the control occurred between 60 and 90 minutes and that at the end of 2 hours the plaques were uncountable.

It is apparent, therefore, that S17 treated with penicillin will absorb either its specific phage or phage to which it is insusceptible. A further interesting point emerges from these counts which clearly show that the presence of penicillin has inhibited the multiplication of phage. Two explanations of this effect are possible. Either, with the concentration of penicillin used, at least 96 p.c. of the cells were killed within an hour and a half and the phage absorbed on to them inactivated; or else there is a more direct action of penicillin in that its bacteriostatic effect on the cells is intimately concerned with alteration of the conditions in the cells necessary for the multiplication of the phage. It has been shown by a number of workers (Dubos, 1945,

p. 317) that penicillin does not completely interrupt protoplasmic growth but prevents cell division. Although it is known that bacteria must be actively metabolizing for phage multiplication to take place within them, there is no evidence to suggest that the actual process of cell division has any connection with phage multiplication. Further experimental work will be necessary to elucidate this point.

## DISCUSSION.

The chief points of interest which emerge from the results reported in this paper are concerned with the behaviour of the lysogenic strains studied, both in their relationships to one another and in the action of penicillin on their phages, and with the effect of penicillin on the ability of the staphylococcal cells to absorb phage.

The fact that an organism carries a phage does not necessarily afford it protection from attack by another phage of the same type. This is shown by the behaviour of the strains S33 and S35, which belong to Type 2A of Wilson and Atkinson; they both carry the same phage and are mutually lysogenic, although they are resistant to filtrates of the phages which they themselves carry. On the other hand, the phage carried may not correspond to the phage type of its host. This is shown by the relationships of S22 and S25, since S22 is not of the same phage type as S25, which its phage attacks. The acquisiton of lysogenicity may therefore be a purely fortuituous phenomenon due to the power of all normal aureus staphylococci to absorb phages to which they are resistant. In the vast majority of cases this absorption results in the inactivation of the phage, but it is possible that in a few cases a symbiosis is established between the absorbed phage and the host cell.

The fact that phage could not be recovered from lysogenic cultures lysed by penicillin may have two explanations. The first would attribute this fact to the intimate relationship between the cell and its phage, as a result of which death of the cell would involve death of the phage. The second explanation could be found in the irreversibility of the union between phage and that part of the bacterial cell to which it is absorbed. It is established that this union takes place between phage and heat-killed cells and also between phage and cell-free extracts of autolysed cells and is obviously, from the speed of its reaction, essentially a surface phenomenon on the intact cell. There is, however, at present no evidence as to the position of the phage in the cell of lysogenic strains, and it is impossible to say which explanation is the correct one.

Little information is available as the mechanism of the action of penicillin on bacteria and it is not certain whether its bacteriostatic, bactericidal and bacteriolytic effects are due to the same process or not. The fact that cells which have been lysed by penicillin do not absorb phage as do the products of autolysis of normal cells or as do penicillin-treated cells which are still intact seems of some significance. The exact chemical nature of the surface component of the staphylococci to which the phage particles are absorbed is not certain, although work done in this laboratory which will be published at a later date indicates that protein is involved in this reaction. It appears that the disruption of the staphylococcal cell, as a result of penicillin action, destroys the surface molecular configuration which is concerned in phage absorption.

## SUMMARY.

The characteristics of five staphylococcal phages derived from lysogenic strains are described.

Penicillin and penicillinase have no demonstrable effect on filtrates of these phages.

Lysis by penicillin of staphylococci carrying phage does not release the phage.

Staphylococci after three hours' contact with penicillin, but not lysed by it, absorb phage as readily as normal staphylococci.

Staphylococci completely lysed by penicillin do not absorb phage.

It is suggested that lysis of the staphylococcus by penicillin completely disrupts the molecular configuration of the cell surface to which phage is absorbed.

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REFERENCES.

Burnet, F. M. (1929): Brit. J. exp. Path., 10, p. 109.
Burnet, F. M. and Lush, D. (1935): J. Path. Bact., Lond., 40, p. 455.
Delbrück, M. (1945): J. Bact., 50, p. 131.
Dubos, R. J. (1945): The Bacterial Cell, Harvard University Press, p. 317.
Fisk, R. T. (1942): J. infect. Dis., 71, p. 153.
Jones, D. (1945): J. Bact., 50, p. 341.
Neter, E. and Clark, P. (1944): *Ibid.*, 48, p. 261.
Todd, E. W. (1945): Lancet, 1, p. 74.
Wilson, G. S. and Atkinson, J. D. (1945): *Ibid.*, 1, p. 647.

# THE INFLUENCE OF FERROUS IRON IN THE DETERMINATION OF ASCORBIC ACID

by F. E. HUELIN AND I. M. STEPHENS

(From the Division of Food Preservation, Commonwealth Council for Scientific and Industrial Research).

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Ascorbic acid in fresh and processed foods is now commonly determined by extraction with 3 p.c. metaphosphoric acid and titration with approximately 0·001 N 2·6 dichlorophenolindophenol. The oxidation of ascorbic acid is promoted both by enzymes and copper in the tissues and metaphosphoric acid preserves the vitamin by inactivating these catalysts. In processed foods the enzymes have already been inactivated by heat, and copper catalysis is generally of greater importance. This effect is probably determined by the concentration of free copper ions, which metaphosphoric acid reduces to a negligible level.

A disadvantage of metaphosphoric acid is that ferrous salts are oxidized quantitatively by the dye in this medium. Canned foods normally contain low concentrations of iron, which is probably retained mostly in the ferrous condition on account of the reducing conditions within the can. A concentration of iron of ten parts per million is quite common, and this could introduce a positive error of 1·6 mg./100 gm. into the determination of ascorbic acid. Errors of this order are significant in assays of many canned foods containing less than 15 mg./100 gm. of ascorbic acid and in studies of ascorbic acid retention in canned foods during storage when the iron content may increase as the ascorbic acid content decreases. A concentration of 50 parts of iron per million, which occurs less frequently, could increase the error to about 8 mg. of ascorbic acid per 100 gm. Higher concentrations, e.g. of the order of 100 parts of iron per million, are encountered only when there is serious internal corrosion of the can, usually associated with hydrogen swelling. Cases have occurred where a high level of iron contamination was derived from the equipment used in processing.

To correct for the presence of ferrous iron in metaphosphoric acid extracts, Lugg (1942) has proposed the use of formaldehyde, which at pH 3·5 condenses rapidly with ascorbic acid, but only slowly with ferrous salts. Hydrogen peroxide has been shown by Marston (1943) and Levy (1943) to eliminate interference by sulphur dioxide. In a pure solution of sulphur dioxide and ascorbic acid, the sulphur dioxide is oxidized rapidly by hydrogen peroxide with only slight attack on the ascorbic acid. The authors have found, however, that when ferrous iron is present in the ascorbic acid solution, the ferrous iron is oxidized by hydrogen peroxide and some of the ascorbic acid is also destroyed. Thus, in extracts containing ferrous iron, the use of hydrogen peroxide converts a positive error into a negative error of less magnitude. Hydrogen peroxide also promotes a slow reoxidation of the reduced dye and the titration should be completed as soon as possible after adding this reagent.

An alternative method of eliminating the interference of ferrous iron is to use extractants other than metaphosphoric acid. Lorenz and Arnold (1938) have shown that ferrous salts titrate with the dye in pure solutions of metaphosphoric or oxalic acid, but not in citric, acetic, phosphoric, sulphuric, hydrochloric, nitric or trichloracetic acids. The effect in metaphosphoric or oxalic acid solutions is probably due to displacement of the ferrous-ferric equilibrium by formation of complex ferric ions, as suggested by Michaelis and Friedheim (1931).

Feaster and Alexander (1944) claim that, if 8 p.c. acetic acid is used for extraction of canned foods, the titre is not affected by ferrous iron up to 100 parts

per million. We have investigated the effect of alternative extractants both on interference by ferrous iron and on the retention of ascorbic acid. Particular attention has been given to acetic and sulphuric acids. These acids reduce interference by ferrous iron, but Ponting (1943) has shown that they are definitely inferior to metaphosphoric and oxalic acids in retention of ascorbic acid.

## EXPERIMENTAL.

*Extraction with Metaphosphoric Acid.*

Ferrous iron interference in metaphosphoric acid extracts and the effect of hydrogen peroxide were first studied. Six canned products—orange juice, tomato juice, broccoli, cabbage, silver beet and French beans—were selected for investigation. Twenty ml. of the liquid portion of each can were diluted to 100 ml. with 3 p.c. metaphosphoric acid. A suitable aliquot (5 ml.) was titrated with approximately 0·001 N dye solution. Similar aliquots were titrated after addition of:

(a) 1 ml. of 1·5 p.c. hydrogen peroxide.
(b) 2 ml. of 0·002 N ferrous ammonium sulphate and,
(c) 2 ml. of 0·002 N ferrous ammonium sulphate, followed by 1 ml. of 1·5 p.c. hydrogen peroxide.

The results were calculated as mg. of ascorbic acid per 100 ml. of the original liquor and are given in Table 1. The added ferrous ammonium sulphate was equivalent to 224 parts per million of ferrous iron or 35·2 mg. of ascorbic acid per 100 ml.

TABLE 1.

*The effect of ferrous iron in metaphosphoric acid extracts.*

| | Apparent ascorbic acid (mg./100 ml.) | | | |
|---|---|---|---|---|
| Product. | Control. | $H_2O_2$. | Ferrous Iron. | Ferrous Iron. + $H_2O_2$. |
| Orange Juice | 33·5 | 32·9 | 69·3 | 30·2 |
| Tomato Juice | 37·0 | 36·7 | 72·1 | 33·0 |
| Broccoli | 38·5 | 37·5 | 71·7 | 28·3 |
| Cabbage | 12·5 | 12·4 | 47·7 | 10·5 |
| Silver Beet | 13·5 | 13·3 | 49·5 | 9·0 |
| French Beans | 6·8 | 6·3 | 41·6 | 3·6 |

The addition of ferrous iron increased the apparent ascorbic acid content by 33–36 mg./100 ml. Hydrogen peroxide alone reduced the ascorbic acid figure by 0·1–1·0 mg./100 ml., but with ferrous iron the loss was increased to 2·0–10·0 mg./100 ml. The iron content of the original products was not more than ten parts per million. It is apparent that hydrogen peroxide cannot be used indiscriminately for elimination of iron interference in metaphosphoric acid extracts. The ascorbic acid figure obtained in the presence of hydrogen peroxide is sufficiently accurate when it is only slightly less than the figure obtained without hydrogen peroxide, *i.e.* when the difference is not more than 1 mg./100 ml. Where the difference is considerable, however, some other method of eliminating the interference of ferrous iron is required.

*Extraction with Acetic and Sulphuric Acids.*

*Interference by ferrous iron.* The effect of alternative extractants on ferrous iron interference was then investigated. Similar dilutions of the canned food liquors were made with 10 p.c. acetic acid and 0·5 N sulphuric acid and 5 ml. aliquots were titrated with and without the addition of 2 ml. of 0·002 N ferrous ammonium sulphate. The results are given in Table 2.

The use of 10 p.c. acetic acid in place of 3 p.c. metaphosphoric acid considerably reduced ferrous iron interference, except in the case of silver beet. Sulphuric acid (0·5 N) practically eliminated this interference in all products except silver beet. With silver beet, interference was still appreciable in 0·5 N sulphuric acid, but much less than in 10 p.c. acetic or 3 p.c. metaphosphoric acid.

The pH values of the extracts were approximately as follows:

3 p.c. metaphosphoric acid, 0·8; 10 p.c. acetic acid, 2·5; 0·5 N sulphuric acid, 0·8.

Ferrous iron does not titrate with the dye in pure solutions of acetic or sulphuric acid and the interference in extracts is probably due to low concentrations of oxalic or other organic acids from the tissues. The worst interference was obtained in extracts of silver beet, which

is known to be high in oxalate. The lesser interference in sulphuric as compared with acetic acid may be due to the effect of pH on the ionisation of organic acids from the tissues and on the formation of ferric complexes by these organic acids.

TABLE 2.

*The effect of ferrous iron in acetic acid and sulphuric acid extracts.*

| Extracting solution. | Product. | Apparent ascorbic acid (mg./100 ml.) Control. | Ferrous Iron. |
|---|---|---|---|
| 10 p.c. Acetic Acid | Orange Juice | 32·4 | 41·7 |
| | Tomato Juice | 36·0 | 42·5 |
| | Broccoli | 37·0 | 38·1 |
| | Cabbage | 10·7 | 11·8 |
| | Silver Beet | 12·7 | 49·0 |
| | French Beans | 4·8 | 6·8 |
| 0·5 N Sulphuric Acid | Orange Juice | 18·4 | 17·3 |
| | Tomato Juice | 35·3 | 35·2 |
| | Broccoli | 36·6 | 35·9 |
| | Cabbage | 10·5 | 10·0 |
| | Silver Beet | 11·8 | 21·4 |
| | French Beans | 5·2 | 5·7 |

To determine the effect of low concentrations of organic acids on ferrous iron interference, investigations were carried out in solutions containing 0·1 M sodium acetate adjusted to various pH levels from 0·4 to 3·0 with sulphuric acid. In these solutions ferrous iron did not react with the dye over the whole range of pH.

When the solutions contained in addition 0·1 M tartrate or citrate, ferrous iron gave appreciable titre at pH 3·0, but not at pH 2·0 or less.

With 0·1 M oxalate, ferrous iron reacted almost quantitatively with the dye over the whole range of pH. At pH 1·0 and above, an appreciable titre was obtained with as little as 0·005 M oxalate present, but at pH 0·4, there was no reaction with 0·02 M oxalate or less.

*Loss of ascorbic acid.* Alternative extractants, such as acetic or sulphuric acid, must ensure adequate retention of ascorbic acid as well as eliminate interference by ferrous iron. To investigate relative loss of ascorbic acid, extracts were titrated (a) immediately and (b) after standing for two hours at laboratory temperature. The results were expressed as percentage loss of ascorbic acid and are given in Table 3.

TABLE 3.

*Loss of ascorbic acid in extracts after two hours.*

| Product. | P.c. loss of ascorbic acid in 3 p.c. metaphosphoric acid (pH 0·8). | 10 p.c. acetic acid (pH 2·5). | 0·5 N sulphuric acid (pH 0·8). |
|---|---|---|---|
| Orange Juice | 2 | 1 | 43 |
| Tomato Juice | 1 | 0 | 30 |
| Broccoli | 4 | 10 | 15 |
| Cabbage | 7 | 5 | 60 |
| Silver Beet | 1 | 4 | 5 |
| French Beans | 22 | 26 | 53 |

Ascorbic acid was retained almost as well in 10 p.c. acetic as in 3 p.c. metaphosphoric acid. Retention in sulphuric acid, however, was very poor. This was quite unexpected in view of the low pH. It was decided to investigate further the effect of pH, copper and other factors on the oxidation of ascorbic acid.

*Effect of copper on the oxidation of ascorbic acid.* Apart from thermolabile enzymes, copper has been found to be the most important catalyst in the oxidation of ascorbic acid. Barron *et al.* (1936) showed that ascorbic acid is not autoxidisable in acid and neutral solutions up to pH 7·0. They investigated the effect of copper, iron, cobalt, nickel, manganese and calcium on the oxidation of ascorbic acid at pH 4–6, and found that copper alone had a marked catalytic effect. Copper catalysis was noticeable with as little as 0·05 parts per million.

We have investigated the oxidation of ascorbic acid in solutions containing 0·1 M sodium acetate adjusted to various pH levels from 0·4 to 3·0 with sulphuric acid. Studies were also made in solutions containing (a) 0·1 M tartrate, (b) 0·1 M citrate, (c) 0·01 M oxalate, or (d) 0·1 M sodium chloride in addition to sodium acetate and similarly adjusted with sulphuric acid. The effect of chloride was investigated because it is commonly used in canning vegetables and because Mapson (1941) has shown that, at laboratory temperatures, low concentrations increase oxidation. All solutions were prepared with glass distilled water.

The oxidation was studied at 40° C. by the following procedure. A 100 ml. florence flask containing 50 ml. of solution was brought to 40° C. in a constant temperature bath. Air was bubbled through at the rate of about 12 ml. per second. After two minutes, 2 ml. of 0·5 p.c. ascorbic acid solution were added. After mixing, 5 ml. of the solution were pipetted into 3 p.c. metaphosphoric acid (to stop oxidation) and titrated with 2·6 dichlorophenolindophenol. Further aliquots were removed at intervals and titrated. The results were expressed as p.c. oxidation of ascorbic acid after five minutes and are given in Table 4. Oxidation was studied without added copper and with the addition of ten parts of copper per million (as copper sulphate). Oxidation in the absence of copper was, however, negligible and the figures in Table 4 refer to the copper-catalysed oxidation.

The results of studies in 0·05 M phthalate buffers are included for comparison. These buffers were adjusted with sulphuric acid to avoid the pro-oxidant effect of chloride.

TABLE 4.

*Oxidation of ascorbic acid (20 mg./100 ml.) in the presence of copper (10 p.p.m.)*

| Buffer. | Added substances. | P.c. oxidation in 5 mins. at pH. 0·4 | 1·0 | 2·0 | 3·0 |
|---|---|---|---|---|---|
| 0·05 M phthalate, adjusted with $H_2SO_4$ | — | — | — | 8·3 | 44·3 |
| 0·1 M sodium acetate, adjusted with $H_2SO_4$. | — | 0 | 0·3 | 7·0 | 54·3 |
| | 0·1 M tartrate | 0 | 0·3 | 2·7 | 8·3 |
| | 0·1 M citrate | 0 | 0·3 | 3·4 | 10·5 |
| | 0·01 M oxalate | 0 | 0 | 0 | 0·3 |
| | 0·1 M NaCl | 0·3 | 6·1 | 81·8 | 84·6 |
| | 0·1 M NaCl + 0·01 M oxalate | 0 | 1·2 | 0·9 | 1·8 |

The copper-catalysed oxidation was not significantly different in phthalate and acetate buffers. Oxidation was somewhat reduced by 0·1 M tartrate or citrate, but much more effectively by 0·01 M oxalate. In the presence of 0·01 M oxalate, oxidation was negligible at pH 0·4–2·0, and only slight at pH 3·0. Chloride (0·1 M) had a marked pro-oxidant effect, which was reduced but still appreciable in the presence of 0·01 M oxalate.

In all solutions, the copper-catalysed oxidation decreased with decreasing pH and hence another explanation is required for the rapid loss of ascorbic acid from sulphuric acid extracts of canned foods, as shown in Table 3.

*Effect of ferrous iron on the oxidation of ascorbic acid.* In the investigations on canned foods, loss of ascorbic acid from sulphuric acid extracts of pH 0·8 was much greater than from acetic acid extracts of pH 2·5. The same relationship was obtained when the drained liquors were diluted and adjusted to various pH levels with sulphuric acid. Canned orange juice was diluted five times and adjusted to pH 0·4, 1·0, 2·0 and 3·0 with sulphuric acid. The oxidation of ascorbic acid in these liquors was studied at 40° C. and expressed as p.c. oxidation in five minutes. The procedure was the same as for the buffer solutions. Measurements were made at each pH (a) without added copper and (b) with ten parts per million of added copper. The results are given in Table 5.

TABLE 5.

*Oxidation of ascorbic acid (20 mg./100 ml.) in diluted canned orange juice.*

| Copper. | P.c. oxidation in 5 mins. at pH. 0·4 | 1·0 | 2·0 | 3·0 |
|---|---|---|---|---|
| No added copper | 13·7 | 3·6 | 0·6 | 0 |
| Added copper (10 p.p.m.) | 2·1 | 1·2 | 10·5 | 17·2 |

In the diluted juice without added copper, the p.c. oxidation was much greater at the lower pH, *i.e.* it was 13·7 at pH 0·4 and nil at pH 3·0. The addition of copper practically reversed the original order. It greatly increased the oxidation at pH 3·0 but reduced it at pH 0·4.

Some catalyst other than copper was obviously responsible for the high rate of oxidation at pH 0·4. The juice was ashed and an ash solution of pH 0·4 and of the same dilution as the original juice was prepared with sulphuric acid. Oxidation of ascorbic acid in this solution was very similar to the original juice. The percentage oxidation in five minutes without added copper was 17·7. With added copper (10 p.p.m.) it was 5·4. The diluted juice and ash solution contained one part per million of iron which appeared to be responsible for the catalytic effect. This was confirmed by adding this concentration of ferrous iron to a similar dilution of fresh orange juice. The per cent. oxidation in this liquor was 13·0 and ten parts per million of copper reduced it to 2·1.

Systematic studies were made of the catalytic effect of ferrous iron using solutions of 0·1 M sodium acetate adjusted to pH 0·4, 1·0, 2·0 and 3·0 with sulphuric acid. The solutions contained ten parts per million of ferrous iron, which were added as ferrous ammonium sulphate. At intervals aliquots were pipetted into 1 N sulphuric acid containing 0·1 p.c. oxalic acid and titrated immediately. This solution was used in place of metaphosphoric acid to avoid interference by ferrous iron. The results were expressed as percentage oxidation after five minutes and are given in Table 6.

The investigations have been confined to ferrous iron, which can be retained in equilibrium with ascorbic acid. Schulek and Floderer (1939) have shown that in acid solution ascorbic acid can reduce ferric iron to the ferrous condition.

TABLE 6.

*Oxidation of ascorbic acid (20 mg./100 ml.) in the presence of ferrous iron (10 p.p.m.)*

(Buffer 0·1 M sodium acetate adjusted with $H_2SO_4$.)

| | P.c. oxidation in 5 mins. at pH. | | | |
|---|---|---|---|---|
| Added substances. | 0·4 | 1·0 | 2·0 | 3·0 |
| — | 43·0 | 20·8 | 0·9 | 0·9 |
| 0·01 M Oxalate | 2·7 | 2·0 | 0·4 | 0·4 |
| 0·1 M NaCl | 13·5 | 3·0 | 0·3 | 0 |
| 0·01 M NaCl + 0·01 M Oxalate | 5·1 | 0 | 0 | 0 |

The iron-catalysed oxidation was considerable at pH 0·4, but only slight at pH 2–3. This was in direct contrast to the copper-catalysed oxidation, which was negligible at pH 0·4 but rapid at pH 2–3. In the presence of 0·01 M oxalate, the iron-catalysed oxidation was reduced, but was still appreciable at pH 0·4. In this case, the rate was actually greater than appears from Table 6. The percentage oxidation in the first five minutes was 2·7, but after fifteen minutes it reached 29·8 as the rate increased considerably during the oxidation. Chloride reduced the catalytic effect of ferrous iron, again in contrast to its effect on copper.

These studies can now be related to the observed stability of ascorbic acid in extracts of canned foods. In solutions containing acetic and sulphuric acids, loss of ascorbic acid at pH 2–3 is largely due to copper catalysis. This effect, however, may be largely suppressed by low concentrations of oxalate or other substances in the tissues. Below pH 1 iron catalysis is of major importance, and this is affected much less by low concentrations of oxalate. The copper content of canned foods is usually only a fraction of the iron content. All these considerations explain why the loss of ascorbic acid was very much greater below pH 1, where iron catalysis predominated, than at pH 2–3, where most of the oxidation is due to copper catalysis.

In the investigations with diluted orange juice, added copper actually reduced the oxidation of ascorbic acid at pH 0·4. This is an effect of copper on iron catalysis, which was also demonstrated in pure solution. At pH 0·4 in the presence of 0·1 M sodium chloride, ten parts per million of ferrous iron gave 13·5 p.c. oxidation of ascorbic acid in five minutes at 40° C. Addition of ten parts per million of copper reduced the oxidation to 4·7 p.c. Thus at pH 0·4 traces of copper have practically no catalytic effect of their own and may actually reduce the catalytic effect of iron.

For comparison of reaction rates, the per cent. oxidation in five minutes rather than a velocity constant has been given, as the reaction kinetics were not similar in all cases. The rate of the copper-catalysed reaction decreased with time, being approximately proportional to the ascorbic acid concentration. That of the iron-catalysed reaction was usually approximately constant. In one case mentioned, the reaction rate increased considerably after the first five minutes, but otherwise the per cent. oxidation in this period formed a satisfactory basis for comparison.

## SUGGESTED PROCEDURES FOR DETERMINING ASCORBIC ACID.

It is suggested that extraction with 3 p.c. metaphosphoric acid is the most suitable routine procedure for canned foods. Aliquots (5 ml.) can be titrated (a) alone and (b) immediately after the addition of 1 ml. of 1·5 p.c. hydrogen peroxide. The second titre is generally lower than the first and can be regarded as affording a sufficiently accurate estimation of ascorbic acid if the two results do not differ by more than 1 mg./100 gm. Otherwise an alternative procedure is required as a check.

An alternative determination by the method of Lugg (1942) is desirable when there is evidence of considerable interference by ferrous iron. It distinguishes "reductones" or apparent ascorbic acid as well as ferrous iron. This, however, may be an advantage as it is in long-stored products that both types of interference are likely to occur. Lugg's method depends on different rates of condensation with formaldehyde.

A procedure using alternative extractants is suggested by us as a result of the investigation described above. The extraction is made with 10 p.c. acetic acid containing 0·1 p.c. of oxalic acid. With silver beet, which is high in oxalate, the oxalic acid can be omitted. The acetic acid extracts have a pH of 2·2–2·5. This is reduced to approximately 0·4 by adding 1 ml. of 5 N sulphuric acid to a 5 ml. aliquot just before titration.

This procedure was tested with canned orange juice, tomato, broccoli, cabbage, silver beet and swede turnip. Addition of iron and copper equivalent to 100 and 10 parts per million respectively in the canned product resulted in a positive error of about 1 mg./100 gm. If the extracts containing iron and copper were allowed to stand for 30 minutes, the error was reduced to practically nil. On further standing the error became negative due to the slow loss of ascorbic acid.

In many canned products the ascorbic acid diffuses to equilibrium within a few days of preparation, and the liquid and solid portions do not differ greatly in ascorbic acid content. The liquid portion can be analysed by a very simple procedure. Pipette 5 ml. of 1 N sulphuric acid into a 20 ml. test tube and bubble carbon dioxide through the solution. Then pipette 1 ml. of liquor from the can immediately after opening and add to the sulphuric acid. Titrate with 2·6 dichlorophenolindophenol. Keep the carbon dioxide bubbling throughout the operation to prevent atmospheric oxidation. By this means the liquor can be brought immediately to a pH sufficiently low to prevent interference.

Where there is evidence of appreciable interference by ferrous iron, it is preferable to use more than one method of estimation. With our present somewhat limited knowledge of interfering substances, checks of this nature are still highly desirable.

SUMMARY.

Ferrous iron introduces a positive error into the determination of ascorbic acid in metaphosphoric acid extracts. Addition of hydrogen peroxide converts this into a negative error of less magnitude.

In acetic and sulphuric acid extracts of canned foods, both iron interference and the stability of ascorbic acid are less at pH 0·4 than at pH 2–3. A similar effect of pH on iron interference is obtained with pure solutions of acetic and sulphuric acid containing 0·01 M oxalate.

The copper-catalysed oxidation of ascorbic acid is negligible at pH 0·4 but considerable at pH 2–3. The iron-catalysed oxidation is considerable at pH 0·4 and only slight at pH 2–3. At pH 0·4 copper actually reduces the iron-catalysed oxidation.

Oxalate (0·01 M) strongly inhibits the copper-catalysed oxidation and partially inhibits the iron-catalysed oxidation. Chloride (0·1 M) increases copper catalysis and reduces iron catalysis.

Various procedures are suggested for determining ascorbic acid in the presence of ferrous iron.

REFERENCES.

Barron, E. S. G. *et al.* (1936): J. biol. Chem., 112, p. 625.
Feaster, J. F. and Alexander, O. R. (1944): Ind. eng. Chem., 36, p. 172.
Levy, L. F. (1943): Biochem. J., 37, p. 713.
Lorenz, A. J. and Arnold, L. J. (1938): Ind. eng. Chem. Anal., 10, p. 687.
Lugg, J. W. H. (1942): Austral. J. exp. Biol., 20, p. 273.
Mapson, L. W. (1941): Biochem. J. 35, p. 1,332.
Marston, H. R. (1943): "The Estimation of Ascorbic Acid"—Austral. chem. Inst. Rpt.
Michaelis, L. and Friedheim, E. (1931): J. biol. Chem., 91, p. 343.
Ponting, J. D. (1943): Ind. eng. Chem. Anal., 15, p. 389.
Schulek, E. and Floderer, I. (1939): Angew. Chem., 52, p. 615.

# THE OCCURRENCE OF SALMONELLA TYPES IN AUSTRALIA.[1] 3.

by NANCY ATKINSON, GWENDOLYN M. WOODROOFE[2] AND A. M. MACBETH.

(From the Institute of Medical and Veterinary Science, Adelaide).

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Continuing our studies on Salmonellas isolated in Australia and its environs, we have investigated another group of cultures, many of which were isolated in Army laboratories. Some of these laboratories were stationed in New Guinea and supplied cultures obtained from sick natives as well as from our soldiers. All the native strains and the majority of those isolated from soldiers seriously ill, proved to be *S. blegdam*, an unusual type of which we had previously found four strains (Atkinson *et al.* 1944) all derived from men who were very ill and had contracted the infection in New Guinea. Other types coming from military hospitals and not previously recorded in Australia were *S. newington*, *S. mississippi*, *S. bareilly*, *S. oslo* and *S. montevideo*. Besides these known types we received from one military hospital a batch of cultures isolated from numerous cases in an epidemic of mild gastro-enteritis. The whole batch proved to be identical but belonged to a new type, which was provisionally called *S. atherton* (Ferris *et al.*, 1945) and was closely related to the $z_4$ paracolons of Edwards *et al.* (1934a). Detailed accounts of these cultures and of the *blegdam* strains will subsequently appear in separate reports.

## ANTIGENIC ANALYSIS OF CULTURES.

The methods used in this work were described in Paper 1 (Atkinson and Woodroofe, 1944) of this series. The tests used for identification of *S. typhi-murium*, *S. blegdam*, *S. bovis-morbificans*, *S. cholerae-suis* var. *kunzendorf*, *S. derby*, *S. paratyphi* B, *S. new-brunswick*, *S. stanley* and *S. reading* appeared in Papers 1 and 2 (Atkinson *et al.*, 1944). A number of types not found in our earlier groups occurred in the present collection of cultures. These types were identified in the following ways:

Strains of *S. newington* (III. XV; eh; 1, 6) fell into Group E of the Kauffmann-White schema by slide agglutination with O antisera. They agglutinated with an absorbed O antiserum specific for XV and thus belonged to the second part of Group E. They were diphasic and in Phase 1 agglutinated with all antisera prepared against organisms containing the factor e and with antiserum specific for the factor h. In Phase 2 tested with antiserum specific for factor 2, 3, 5, 6 or 7 agglutination occurred only with antiserum 6. A representative culture also completely absorbed the H agglutinins from *S. anatum* (III, X, XXVI: eh; 1, 6) antiserum.

Strains of *S. mississippi* (I, XIII, XXIII; b; 1, 5) agglutinated with O antisera prepared against organisms containing the factor XIII; they also agglutinated with absorbed O antiserum specific for I or XXIII. They were diphasic and in Phase 1 agglutinated with antiserum specific for the factor b; in Phase 2 they agglutinated in antiserum specific for the factor 5. They failed to react with other absorbed H antisera.

By slide agglutination with O antisera the strain identified as *S. oslo* (VI, VII; a; enx) fell into Group C of the Kauffmann-White schema. It agglutinated with O antiserum specific for VII, but failed to react with that specific for VIII. The culture was diphasic with the factor e (not 1) in the second phase; Phase 1 contained the antigen a and Phase 2 agglutinated with antiserum specific for x.

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[2]Working with a grant from the University of Adelaide.

The strain identified as *S. montevideo* (VI, VII; gms; —) belonged to Group C by slide agglutination with O antisera and agglutinated with O antiserum specific for VII, but failed to react with that specific for VIII. It was monophasic and agglutinated with all antisera prepared against organisms with the H factor g or m. In antiserum specific for f, p, g, m or s it agglutinated only with m and s. Finally it absorbed all the H and O agglutinins from *S. montevideo* antiserum.

The strain of *S. bareilly* (VI, VII; y; 1, 5) agglutinated with O antisera for Group C and with O antiserum specific for VII; it did not react with O antiserum specific for VIII. It was diphasic and possessed antigen y in Phase 1 and antigen 5 in Phase 2.

The strain *S. enteritidis* ([I], IX, XII; gm; —) possessed somatic antigen IX but did not react with O antiserum specific for I. It was monophasic and agglutinated with all antisera prepared against organisms containing the H factor g. With absorbed antisera for the other factors associated with g in the *enteritidis* group agglutination occurred only in that for factor m.

The characteristics of *S. atherton* will be described in detail in a subsequent paper.

## THE ORIGIN AND FREQUENCY OF OCCURRENCE OF THE SALMONELLA TYPES.

The present group of cultures contained 156 strains of which 54 were *S. typhi-murium*, 27 *S. blegdam*, 31 *S. atherton*, 15 *S. bovis-morbificans*, 6 *S. cholerae-suis* var. *kunzendorf*, 5 *S. newington*, 4 *S. derby*, 3 *S. new-brunswick*, 2 *S. stanley*, 2 *S. mississippi*, 2 *S. paratyphi* B, and one each of *S. reading*, *S. bareilly*, *S. enteritidis*, *S. oslo* and *S. montevideo*.

All strains of *S. typhi-murium* were tested in Bitter medium for fermentation of maltose, xylose and rhamnose. The results appear in Table 1 along with the analysis of the somatic antigens.

TABLE 1.

*Source and reactions of strains of* S. typhi-murium.

| Source of strain. | No. of strains found. | Bitter test Maltose. | Xylose. | Rhamnose. | Somatic antigens. |
|---|---|---|---|---|---|
| Children with gastro-enteritis (faeces) | 2 | + | + | + | I, IV, V |
| | 5 | + | ± | + | I, IV, V |
| | 1 | + | — | + | I, IV, V |
| | 3 | ± | — | + | I, IV, V |
| | 1 | + | + | — | I, IV, V |
| | 6 | + | + | + | IV, V |
| | 7 | ± | + | + | IV, V |
| | 1 | + | ± | + | IV, V |
| | 3 | + | — | + | IV, V |
| | 1 | — | ± | ± | IV, V |
| | 1 | — | — | + | IV, V |
| | 1 | + | — | — | IV, V |
| | 1 | + | — | + | IV |
| | 1 | — | — | + | IV |
| Children with meningitis (C.S.F. brain and spleen) | 4 | + | — | + | I, IV, V |
| Adults with gastro-enteritis (faeces, lung) | 1 | ± | + | + | I, IV, V |
| | 1 | + | — | + | I, IV, V |
| | 1 | ± | — | — | I, IV, V |
| | 2 | + | + | + | IV, V |
| | 3 | + | — | + | IV, V |
| | 1 | ± | ± | ± | IV, V |
| | 2 | + | — | — | IV |
| Guinea-pigs with epidemic disease (spleen) | 1 | + | + | + | I, IV, V |
| | 3 | ± | — | + | IV |
| Flummery causing gastro-enteritis | 1 | + | ± | + | IV, V |

This Table shows that by far the largest number of strains came from infections in children and were the IV, V type with or without I; these strains with few exceptions, fermented maltose and rhamnose in the Bitter test but nearly one-third of them, including those from the two cases of meningitis, failed to attack xylose. Only two IV-variants occurred among the children's cultures; neither fermented xylose and one failed to ferment maltose. The two

IV-variants found among the adults' strains and the three from guinea-pigs also showed poor fermentative activity. In a group of cultures of *S. typhi-murium* from various animals, Edwards and Bruner (1940) recorded variation in xylose fermentation for their full antigenic types. They did not find any IV-variants among their guinea-pig cultures, but we have found three of these variants out of four strains isolated from diseased guinea-pigs. These three cultures came from three different animals all belonging to the stock of a single laboratory and their Bitter reactions were identical.

Table 2 summarizes information on the origin and effect of the present group of Salmonellas.

TABLE 2.

*Salmonellas described in this paper.*

| Salmonella. | Total number of strains. | Where isolated. | Source. | No. of strains. | Type of illness. |
|---|---|---|---|---|---|
| *S. typhi-murium* | 54 | South Australia | Children (faeces) | 26 | Gastro-enteritis |
| | | | Adult (faeces) | 1 | Gastro-enteritis |
| | | Victoria. | Children (faeces) | 7 | Gastro-enteritis |
| | | | Adults (faeces) | 3* | Gastro-enteritis |
| | | | Adult (lung) | 1 | Acute gastro-enteritis ending fatally |
| | | Queensland | Child Cerebrospinal fluid) | 1 | Meningitis |
| | | | Child Cerebrospinal fluid, brain, spleen) | 3 | Meningitis ending fatally |
| | | | Guinea-pig (spleen) | 2* | Epidemic disease in laboratory stock. |
| | | | Guinea-pig (spleen) | 2* | From apparently clean stock. |
| | | | Adults (faeces) | 2* | Gastro-enteritis |
| | | | Adult (faeces) | 1* | Enteric-like fever |
| | | | Adult (faeces) | 1* | Clinically dysentery |
| | | | Adult (faeces) | 1* | Salmonella fever (very toxic) |
| | | | Flummery | 1 | Eaten by nine persons all of whom contracted gastro-enteritis |
| | | New South Wales | Adult (faeces) | 1* | Clinically dysentery |
| | | | Child (faeces) | 1 | Gastro-enteritis |
| *S. blegdam* | 27 | New Guinea and Bougainville Island | Australian soldiers (blood and faeces) | 15* | Enteric-like fever with or without complications |
| | | | Natives of these Islands | 5* | Enteric-like fever and other manifestations, one healthy carrier |
| | | | Japanese prisoner of war | 1* | Enteric-like fever (fatal) |
| | | Victoria | Mice | 5* | Epidemic disease in laboratory stock |
| | | | Guinea-pig (blood) | 1* | Epidemic disease in laboratory stock |
| *S. atherton* | 31 | Queensland | Adults (faeces) | 31* | All strains from a single outbreak of gastro-enteritis |
| *S. bovis-morbificans* | 15 | South Australia | Children (faeces) | 9 | Gastro-enteritis |
| | | Victoria | Child (faeces) | 1 | Gastro-enteritis |
| | | Queensland | Child (faeces) | 1 | Gastro-enteritis |
| | | | Adults (faeces) | 4* | Acute gastro-enteritis |
| *S. cholerae-suis* var. *kunzendorf* | 6 | South Australia | Adult (synovial fluid) | 1 | Arthritis |
| | | | Pigs (blood) | 3 | Fever |
| | | | Pig (muscle) | 1 | Suspected of swine erysipelas |
| | | Victoria | Adult (faeces) | 1* | Abdominal colic, diarrhoea |

TABLE 2 (continued).

| Salmonella. | Total number of strains. | Where isolated. | Source. | No. of strains. | Type of illness. |
|---|---|---|---|---|---|
| *S. newington* | 5 | Queensland | Adults (faeces) | 3* | Gastro-enteritis |
| | | | Adult (faeces) | 1* | Enteric-like fever |
| | | Northern Territory | Adult (faeces) | 1* | Gastro-enteritis |
| *S. derby* | 4 | South Australia | Children (faeces) | 3 | Gastro-enteritis |
| | | Northern Territory | Adult (faeces) | 1* | Gastro-enteritis |
| *S. new-brunswick* | 3 | Queensland | Adults (faeces) | 2* | Outbreak of severe food-poisoning involving about 30 cases |
| | | | Adult (faeces) | 1* | Mild diarrhoea |
| *S. stanley* | 2 | South Australia | Child (faeces) | 1 | Gastro-enteritis |
| | | | Adult | 1* | No history available |
| *S. mississippi* | 2 | Queensland | Adults (faeces) | 2* | Outbreak of gastro-enteritis in a single ward, involving 9 cases |
| *S. paratyphi* B | 2 | South Australia | Adult (faeces and duodenal contents) | 2 | Paratyphoid carrier |
| *S. reading* | 1 | New Guinea | Adult (faeces) | 1* | Gastro-enteritis |
| *S. bareilly* | 1 | South Australia | Adult (faeces) | 1* | Gastro-enteritis |
| *S. enteritidis* | 1 | Victoria | Guinea-pig (spleen) | 1 | Epidemic disease in laboratory stock |
| *S. oslo* | 1 | Queensland | Adult (faeces) | 1* | Recurrent abdominal pains and diarrhoea |
| *S. montevideo* | 1 | Morotai | Adult (faeces) | 1* | Gastro-enteritis |

* These strains were obtained solely or mainly from military hospitals.

By far the commonest type was *S. typhi-murium* and the main manifestation of its infection in either adults or children was gastro-enteritis. Occasionally a much more severe illness occurred; in our series two children had meningitis and one died; one adult died after acute gastro-enteritis. Four strains came from epidemic or latent infections in laboratory stocks of guinea-pigs.

In Paper 2 of this series (Atkinson *et al.*, 1944) we recorded the occurrence of *S. blegdam* in blood and faeces of four cases of enteric-like fever contracted in New Guinea. Now we record the finding of 21 more human strains, the majority from cases of severe enteric-like fever, often with complications, occurring in New Guinea and Bougainville Island. Six animal strains were isolated in Australia from mice and guinea-pigs in laboratory stock suffering from an epidemic. A preliminary account of all these strains has appeared elsewhere (Atkinson, 1946).

With the exception of the strains of *S. cholerae-suis* var. *kunzendorf*, *S. enteritidis* and *S. paratyphi* B, the other Salmonellas came from faeces of children or adults suffering from gastro-enteritis.

The two strains of *S. paratyphi* B both came from the same individual, one from the faeces, the other from duodenal contents. Serologically these strains appeared identical and contained O antigens I, IV and V. The faecal strain, however, differed from the other in producing only traces of gas in fermentation tests with glucose, mannitol and maltose. At the time of our investigation the patient's serum agglutinated a specific suspension of *S. paratyphi* B to a titre of 1/320.

The one strain of *S. enteritidis* came from the spleen of a guinea-pig dying of an epidemic disease in laboratory stocks.

Of our six strains of *S. cholerae-suis* var. *kunzendorf* four came from blood or muscle of pigs slaughtered at the abattoirs and condemned by the meat inspectors; one was tentatively described as a case of swine erysipelas, but no *Erysipelothrix* was detected. The other two strains came from human cases, one of which showed intestinal symptoms which cleared up

after removal of the appendix; the other case (one of arthritis) was first recorded in Paper 2 of this series when the organism was isolated from synovial fluid from the knee-joint. The second isolation of the organism was made nearly a year later from synovial fluid from the same knee-joint *post mortem*. The interesting feature of this second strain of *S. cholerae-suis* var. *kunzendorf* was its mucoid form in which it showed none of the typical H or O antigens. Reversion to the non-mucoid form quickly occurred in broth culture and the organism was then easily identified by its flagellar and somatic antigens. Both mucoid and non-mucoid strains showed abundant production of $H_2S$, a reaction which, according to Bruner and Edwards (1939), characterized the nonspecific monophasic type.

## DISCUSSION.

Among this group of 156 strains of Salmonella several types new to Australia were discovered, namely *S. mississippi*, *S. newington*, *S. oslo*, *S. montevideo*, *S. bareilly*; a new type tentatively named *S. atherton*, also occurred and was closely related to the $z_4$ paracolons and the Salmonellas such as *S. arizona*. Thirty-one strains of this new type were obtained from an epidemic of gastro-enteritis of which Ferris *et al.* (1945) recorded the details. There seemed little doubt that the organism was responsible for the disease; sera from the majority of patients agglutinated an O suspension, but sera from normal individuals failed to do so. If *S. arizona* is acceptable as a Salmonella (see Kauffmann, 1941), then there should be no obvious reason for excluding our new type. However, the details of cultural reactions and antigenic analysis will appear elsewhere along with a discussion of this problem.

Edwards *et al.* (1943b), in the United States, first described *S. mississippi*, one strain of which was isolated from the stool of an apparently normal food-handler. Our two strains came from faeces of two cases of gastro-enteritis of which a total of nine cases occurred within three days in a single ward in a military hospital. Clearly this organism is a human pathogen. It was isolated from the stools of all nine cases.

*S. montevideo* was first isolated and described by Hormaeche and Salsamendi (1936) and Hormaeche and Peluffo (1936) in South America; they obtained strains from both human and animal material, including mesenteric glands of healthy pigs. Schiff and Saphra (1940) in U.S.A. found a strain in faeces of a patient with gastro-enteritis; Kauffmann (1941) recorded cultures isolated in Denmark and in Tel-Aviv. Our strain from a case of gastro-enteritis, was isolated in Morotai and provides further evidence for the widespread distribution of the type. Bornstein (1943) recorded 34 strains identified at the New York Centre and obtained from cases of septicaemia, pyrexia or gastro-enteritis in North America or Cuba. Edwards and Bruner (1943) recorded 21 cultures from man (of which 8 came from carriers) and 17 strains from animals, mainly fowls. D'Albora *et al.* (1945), described an outbreak of food-poisoning due to *S. montevideo* in an American Army Hospital. Our case showed very acute illness with high temperature, but blood cultures were negative and recovery occurred within a week.

*S. newington* is closely related to *S. anatum* and the first recorded cultures were confused with this type. Edwards (1937) cleared up the confusion and showed that, although both organisms possessed the same flagellar antigens in Phases 1 and 2, they could be distinguished by their somatic antigens, in spite of the fact that both contain the factor III; *S. anatum* (III, X, XXVI; eh; 1, 6) also possessed X and XXVI neither of which occurred in *S. newington* (III, XV; eh; 1, 6) which contained XV, a factor absent in *S. anatum*. *S. new-brunswick* (III, XV; lv; 1, 7) which we first recorded for Australia in Paper 2 of this series (one strain only) and which we again record in the present series (three strains) has the same O antigens as *S. newington* and was defined by Edwards in the same paper. Both these types were first isolated from birds and have subsequently been found causing outbreaks of disease in turkeys (Hinshaw *et al.* 1943, 1944). *S. newington* seems commoner than *S. new-brunswick* in man; Edwards and Bruner (1943) listed 31 out of a total of 65 strains of *S. newington* as derived from human carriers or cases of gastro-enteritis, whereas no human strains of *S. new-brunswick* appeared out of their total of 43. We found five strains of *S. newington*, all from gastro-enteritis in soldiers. Bornstein (1943) recorded neither organism among those found by the New York Centre. That *S. new-brunswick* is capable of infecting man is shown by our four isolations, two of which came from an outbreak of gastro-enteritis involving about 30 individuals. Kauffmann (1941) recorded a couple of cases of infection with this organism.

Infections in man with *S. bareilly* are not uncommon. Bornstein (1943) included 13 strains among the 500 typed at the New York Centre; three of these came from septicaemias and one patient died. Edwards and Bruner (1943) found 72 strains out of a total of 3,090; the majority of these strains came from poultry, but 13 were isolated from human cases of gastro-

enteritis or carriers. Our case of infection with *S. bareilly* was a typical gastro-enteritis; serum from the patient agglutinated specific H suspensions of the autogenous strain and the stock strain. The original isolation and description of *S. bareilly* was made from mild cases of enteric fever in India (see Kauffmann, 1941).

Tesdal (1937) first isolated and described *S. oslo* from a case of acute gastro-enteritis whose serum agglutinated both H and O suspensions of the organism. According to Bornstein (1943) no other strains have been isolated. Therefore we give in some detail the description of our case which occurred in an Australian soldier. In Syria, two and a half years earlier he contracted dysentery and since the first attack suffered from recurrent bouts of abdominal pain with diarrhoea. Our strain of *S. oslo* was isolated from his faeces while in hospital during a prolonged attack accompanied by a relapse of malaria. After recovery his faeces were examined regularly for four weeks and *S. oslo* was not detected. No report on the bacteriological examination of stools was available to confirm the diagnosis of his Syrian attack as bacillary or amoebic dysentery. The possibility remains that this condition was due to infection with *S. oslo*. We found only this one strain among all the Salmonellas we have so far typed.

The number of strains of the rare type *S. blegdam* has risen from four in our Paper 2 to 27 in the present report. Now there is no doubt that this organism is responsible for severe enteric-like fever in man, often with complications, but the infection occurs, not in Australia, but in New Guinea and adjacent islands. Details of cases and a preliminary report on the isolated cultures appeared in articles by Fenner and Jackson (1946) and Atkinson (1946). Gastro-enteritis was not a manifestation of *S. blegdam* infection.

In accord with our previous findings, *S. typhi-murium* was the commonest Salmonella and provided 54 strains out of the total of 156. The IV-variant was rarely found (seven out of 54) and three, out of a total of seven, came from guinea-pigs not humans; all showed variable fermentation powers in the Bitter tests but all gave negative results with xylose. Strains from a single source, for example guinea-pigs from one laboratory, produced identical reactions. The full antigenic types varied in ability to attack xylose in the Bitter test and thus fell into two main groups; the tests in maltose and rhamnose were mainly positive. The results of our Bitter tests were much the same as those of Edwards and Bruner (1940) who found many rhamnose positive, xylose and maltose negative strains among their IV-variants; among strains of the full antigenic type the majority were maltose and rhamnose positive but variable in xylose. The results of their Bitter tests and the antigenic type were epidemiologically significant. Although *S. typhi-murium* usually causes gastro-enteritis in man, occasionally it produces severe infection; among the present series we recorded two cases of meningitis in infants, one of whom died. The organism was isolated from cerebrospinal fluid in both cases, and in the fatal case from brain and spleen. Bornstein (1943) and Kauffmann (1941) recorded *S. enteritidis* as the commonest type found in meningitis which practically always occurred in infants under one year of age; however, *S. typhi-murium* and some of the rare types were found occasionally. One adult admitted to hospital with severe gastro-enteritis died and the organism was isolated from lung post-mortem. No further details were available, but Cleland (1944) recorded the results of the autopsy on another case of *S. typhi-murium* infection in an adult. He found only slight congestion of the intestinal tract; the organism was isolated from the liver. Death from such an infection seems to be unaccompanied by gross pathological changes. Two of our cases in soldiers were described by the medical officer as enteric-like or Salmonella fever which was very toxic, and two more appeared clinically to be dysentery.

Typical gastro-enteritis occurred in both adults and children from whom strains of *S. bovis morbificans, S. derby, S. stanley* and *S. reading* were isolated. All these types have been recorded before for Australia in Paper 1 or 2 of this series.

The strain of *S. cholerae-suis* var. *kunzendorf* obtained *post-mortem* from synovial fluid from the knee was a mucoid variant. Some months previously, however, the same patient had provided, from fluid from the same knee, a nonmucoid culture of the organism; this strain was recorded in Paper 2 of this series. At autopsy no Salmonella was detected in the internal organs or the intestinal tract and the cause of death was given as pneumonia. Various Salmonellas can be induced to produce a mucoid phase in the "slime-wall" phenomenon, and Kauffmann (1941) considered the typical M antigen (Schleimantigen) to be practically the same for all. According to Bornstein (1943) mucoid variants of *S. paratyphi* B have been found in faeces of carriers, but naturally occurring mucoid variants of other types were very rare. We detected no mucoid variants in the faeces or duodenal contents of our carrier of *S. paratyphi* B. Twelve years previously this patient had suffered from paratyphoid fever, which had been followed a year later by removal of the gall-bladder; the patient remained a carrier. This case emphasises the difficulty in clearing a carrier of this organism.

As compared with figures for other parts of the world our group of Salmonellas showed a high percentage of the so-called rare types. Kauffmann (1941) described 1,262 strains isolated in England, Denmark and Germany, of which 65 p.c. were *S. typhi-murium*, 16 p.c. were types of Group C, 14 p.c. were types of Group D, and only 5 p.c. belong to the other groups including Group B (without *S. typhi-*

*murium*). Out of 500 human strains from North America and Cuba, Bornstein (1943) recorded 158 *S. typhi-murium* and 78 other strains of Group B, 188 types of Group C, 38 types of Group D, and 39 types of Group A, Group E and the miscellaneous Group F in which many of the rare types are placed. Out of our 156 strains 54 were *S. typhi-murium* and 9 were other members of Group B, 24 belonged to Group C, 28 to Group D, and 10 to Groups E and F together; the remaining 31 strains were *S. atherton*, the new type related to the $z_4$ paracolons. All our Group D strains, except one, were *S. blegdam*, a very rare type, and, therefore, we may say that out of 156 strains examined at least 68 proved to be uncommon types.

## SUMMARY.

A further group of 156 cultures of Salmonellas were typed at the Adelaide Typing Centre. Many of the cultures were obtained from Australian Army hospitals and all were isolated in Australia or the neighbouring islands.

There were 26 strains of the rare type *S. blegdam* which caused enteric-like fever in soldiers in New Guinea.

Thirty-one strains of a new type, tentatively named *S. atherton*, were obtained from a single outbreak of gastro-enteritis in military personnel. This type was closely related to the $z_4$ paracolons.

Other types recorded for the first time for Australia and its environs were *S. mississippi*, *S. newington*, *S. oslo*, *S. montevideo*, and *S. bareilly*.

Excepting *S. blegdam*, the main manifestation of infection with a Salmonella was gastro-enteritis, but two cases of *S. typhi-murium* meningitis occurred in infants.

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## REFERENCES.

Atkinson, N. (1946): Med. J. Austral., 1, p. 326.
Atkinson, N. and Woodroofe, G. M. (1944): Austral. J. exp. Biol., 22, p. 51.
Atkinson, N., Woodroofe, G. M. and Macbeth, A. M. (1944): *Ibid.*, 22, p. 201.
Bornstein, S. (1943): J. Immunol., 46, p. 439.
Bruner, D. W. and Edwards, P. R. (1939): Amer. J. Hyg., 30, p. 75.
Cleland, J. B. (1944): Med. J. Austral., 2, p. 59.
D'Albora, J. B., Ingegno, A. P. and Edson, J. N. (1945): J. Amer. med. Assn., 129, p. 10.
Edwards, P. R. (1937): J. Hyg. Camb., 37, p. 384.
Edwards, P. R. and Bruner, D. W. (1940): University of Kentucky, Bulletin No. 400.
Edwards, P. R. and Bruner, D. W. (1943): J. infect. Dis., 72, p. 58.
Edwards, P. R., Cherry, W. B. and Bruner, D. W. (1943a): J. infect. Dis., 73, p. 229.
Edwards, P. R., Cherry, W. B. and Bruner, D. W. (1943b): Proc. Soc. exp. Biol. N.Y. 54, p. 263.
Fenner, F. and Jackson, A. V. (1946): Med. J. Austral., 1, p. 313.
Ferris, A. A., Hertzberg, R. and Atkinson, N. (1945): *Ibid.*, 2, p. 368.
Hinshaw, W. R. and McNeil, E. (1943): Poultry Science, 22, p. 415.
Hinshaw, W. R., McNeil, E. and Taylor, T. J. (1944): Amer. J. pub. Health, 40, p. 264.
Hormaeche, E. and Peluffo, C. A. (1936): Arch. Urug. Med. Cir. Espec., 9, p. 673 (cited by Kauffmann, 1941).
Hormaeche, E. and Salsamendi, R. (1936): *Ibid.*, 9, p. 665 (cited by Kauffmann, 1941).
Kauffmann, F. (1941): "Die Bakteriologie der Salmonelle-Gruppe," Kopenhagen, Einer Munksgaard.
Schiff, F. and Saphra, I. (1940): J. infect. Dis., 66, p. 97.
Tesdal, M. (1937): Z. Hyg., 119, p. 451.

# CYSTINE, METHIONINE AND SULPHATE EXCRETION IN CYSTINURIA

by JOAN B. CLELAND

(From the Institute of Medical and Veterinary Science, Adelaide).

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Cysteine and methionine are now considered to be the chief sources of urinary cystine in cystinuria. However, the excretion of extra cystine following the ingestion of given amounts of these amino-acids is variable and Hess and Sullivan (1942) have reported a case in which cystine excretion was increased when either glycine or alanine were taken, and Cleland (1946) obtained extra cystine after giving alanine (but not glycine) to a cystinuric. This paper gives the results obtained when leucine, serine, alanine, and glycine, as well as some of the sulphur-containing amino acids, were given to a cystinuric.

## EXPERIMENTAL DETAILS.

The cystinuric patient was the young woman on whom some investigations had already been carried out (Cleland, 1945). Since the completion of these earlier studies frequent X-ray examinations have shown no evidence of new stone formation in the kidney although the urine continues to contain cystine crystals.

*Diet.* During these experiments the patient, who was working, consumed her normal diet which usually consisted each day of an egg, one serve of meat, a pint of milk, cereals, fruit and vegetables. The total protein intake was about 1·2 gm. per kilo.

*Amino acid supplements.* The amino acid supplements were taken in gelatine capsules on the morning of the day indicated. In one series of experiments the supplement consisted of 2·5 gm. methionine together with the molecular equivalent of leucine, serine, glycine or alanine taken on two consecutive days. In the other series the supplement was 1 gm. cystine or its equivalent of methionine or homocystine, 1·3 gm. serine, alanine or glycine or 2·2 gm. leucine each taken on only one occasion.

The dl-serine was obtained from Gelatin Products through the kindness of Dr. E. Ellion of Washington, and the dl-homocystine through the courtesy of Dr. V. du Vigneaud of Cornell University. Cystine was isolated from wool and the methionine, glycine and alanine came from L. Light and Co. Ltd., May and Baker and B.D.H. respectively.

*Methods.* The methods used were those described in previous papers (Cleland, 1945 and 1946).

## RESULTS AND DISCUSSION.

Results are shown in Tables 1 and 2 and Fig. 1.

The excretions of extra cystine, methionine and sulphate sulphur following the ingestion of the amino acids were calculated for the days on which they were fed and the following day. Normal values were obtained from the one or two days preceding the test.

The results have also been expressed as the ratio of cystine, methionine and sulphate sulphur excreted per gm. of nitrogen (Fig. 1).

In comparing the effects of equivalent amounts of amino acids it should be remembered that where the nitrogen excretion is low (indicating reduced protein catabolism) values for the recovery of extra sulphur will tend to be low and the excretion of sulphur fractions per gm. of nitrogen will tend to be high while the reverse conditions hold when the nitrogen excretion is high.

The results can be discussed under three headings.

### *1. The Effect of Single Small Doses of Sulphur-Containing Amino Acids.*

*Cystine.* As was found previously, the ingestion of cystine was followed by a marked rise in urinary sulphate. The extra sulphate excreted on this occasion was, however, greater than could be accounted for by the complete oxidation of the cystine, and there was also a small rise in methionine excretion. This exaggerated effect was probably due to the concurrent increase in urinary nitrogen—the average excretion being 10·2 gm. N per day after cystine

and 9 gm. per day for the two preceding days. The effect of cystine can therefore be better shown when the results are expressed as cystine, methionine and sulphate sulphur excreted per gm. of nitrogen (Fig. 1). This shows a definite rise in the ratio of urinary sulphate to total nitrogen, an unaltered methionine: nitrogen ratio and a relative fall in cystine excretion.

These results are similar to those obtained from the same cystinuric about 18 months previously and show that ingested cystine is not a source of extra urinary cystine (cystine excretion tends to fall) but is almost completely oxidized to sulphate.

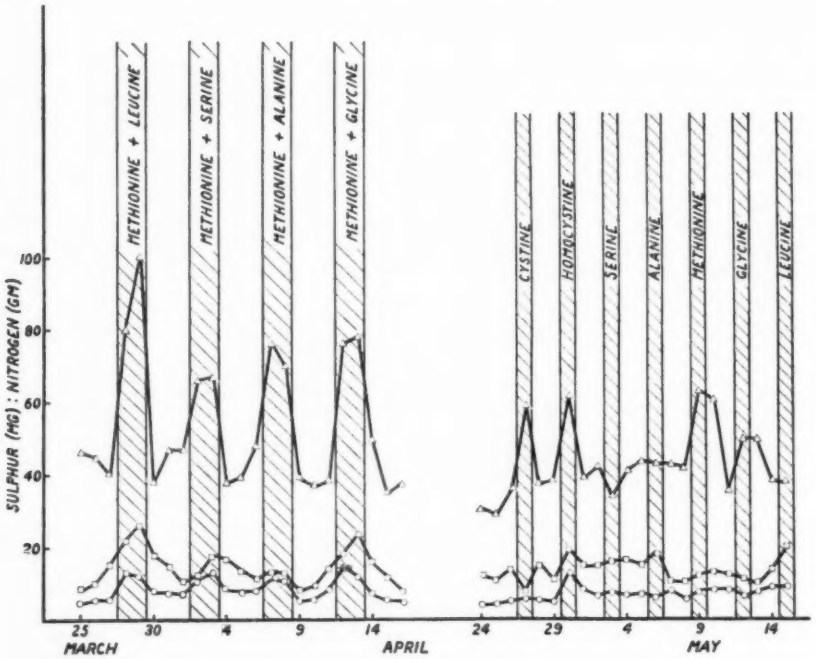


**Fig. 1.** Ratio of urinary sulphate, cystine and methionine sulphur to total urinary nitrogen excreted each day.

△ Sulphate sulphur (mg.): Total nitrogen (gm.).
□ Cystine sulphur (mg.) : Total nitrogen (gm.).
O Methionine sulphur (mg.): Total nitrogen (gm.).

*Methionine.* The ingestion of an equivalent amount of methionine was also followed by a great increase in sulphate excretion but, in contrast to the effect of cystine, this was accompanied by a rise in urinary cystine and methionine. The extra sulphate excreted was greater than could be accounted for by the complete oxidation of the ingested amino acid, and this again can be attributed to concurrent variations in nitrogen excretion, the average being in this case 8·8 gm. N per day after methionine and 7·2 gm. N for the preceding day. The ratio of urinary sulphate sulphur to total nitrogen (Fig. 1) after methionine was increased to about the same level as after an equivalent amount of cystine, but there was also a small rise in both cystine and methionine excretion per gm. of nitrogen, the relative rise in sulphate being greater and in cystine and methionine lower than the results obtained 18 months before (Cleland, 1945). This difference is not unexpected for others (Lewis *et al.*, 1936; Lough *et al.*, 1941) have shown that the excretion of extra cystine following a given quantity of methionine tends to decrease with increasing protein intake, and the average N excretion during the earlier experiment was only 4·8 gm. per day.

These results confirm those obtained from the same cystinuric on a previous occasion in which methionine was found to increase cystine, methionine and sulphate excretion (Cleland, 1945).

TABLE 1.

*Influence of various amino acids on urinary sulphur in cystinuria.*

| Date. | Amino acid supplement. | Volume (ml.) | Total N (gm.) | Sulphur (gm.) as Cystine. | Methionine. | Sulphate. |
|---|---|---|---|---|---|---|
| March 25 | | 650 | 7·6 | 0·06 | 0·04 | 0·36 |
| 26 | | 820 | 8·2 | 0·08 | 0·05 | 0·36 |
| 27 | | 1,350 | 7·9 | 0·13 | 0·04 | 0·32 |
| 28 | 2·5 gm. methionine + 2·2 gm. leucine | 1,130 | 7·5 | 0·17 | 0·10 | 0·60 |
| 29 | 2·5 gm. methionine + 2·2 gm. leucine | 1,100 | 6·9 | 0·18 | 0·09 | 0·70 |
| 30 | | 1,570 | 9·8 | 0·17 | 0·08 | 0·37 |
| 31 | | 1,070 | 7·5 | 0·12 | 0·06 | 0·36 |
| April 1 | | 720 | 8·0 | 0·09 | 0·06 | 0·37 |
| 2 | 2·5 gm. methionine + 1·75 gm. serine | 1,000 | 10·0 | 0·13 | 0·11 | 0·67 |
| 3 | 2·5 gm. methionine + 1·75 gm. serine | 1,170 | 10·6 | 0·19 | 0·14 | 0·72 |
| 4 | | 1,610 | 9·4 | 0·16 | 0·08 | 0·36 |
| 5 | | 900 | 6·6 | 0·09 | 0·05 | 0·27 |
| 6 | | 1,050 | 8·8 | 0·10 | 0·07 | 0·42 |
| 7 | 2·5 gm. methionine + 1·5 gm. alanine | 1,070 | 10·7 | 0·15 | 0·12 | 0·82 |
| 8 | 2·5 gm. methionine + 1·5 gm. alanine | 750 | 8·9 | 0·12 | 0·10 | 0·62 |
| 9 | | 600 | 8·8 | 0·07 | 0·05 | 0·34 |
| 10 | | 750 | 10·0 | 0·10 | 0·06 | 0·37 |
| 11 | | 1,160 | 8·5 | 0·12 | 0·07 | 0·34 |
| 12 | 2·5 gm. methionine + 1·25 gm. glycine | 1,160 | 8·8 | 0·16 | 0·13 | 0·62 |
| 13 | 2·5 gm. methionine + 1·25 gm. glycine | 1,760 | 10·5 | 0·24 | 0·13 | 0·82 |
| 14 | | 630 | 7·3 | 0·11 | 0·05 | 0·37 |
| 15 | | 600 | 8·7 | 0·11 | 0·05 | 0·31 |
| 16 | | 620 | 9·1 | 0·08 | 0·05 | 0·34 |
| 24 | | 1,100 | 10·2 | 0·13 | 0·04 | 0·32 |
| 25 | | 870 | 9·2 | 0·11 | 0·04 | 0·27 |
| 26 | | 1,200 | 8·8 | 0·13 | 0·05 | 0·31 |
| 27 | 1 gm. cystine | 720 | 10·4 | 0·08 | 0·05 | 0·61 |
| 28 | | 1,450 | 10·0 | 0·16 | 0·06 | 0·38 |
| 29 | | 1,050 | 8·8 | 0·10 | 0·05 | 0·34 |
| 30 | 1·1 gm. homocystine | 850 | 6·3 | 0·12 | 0·09 | 0·39 |
| May 1 | | 1,150 | 8·4 | 0·13 | 0·07 | 0·33 |
| 2 | | 1,150 | 7·5 | 0·11 | 0·05 | 0·32 |
| 3 | 1·3 gm. serine | 1,020 | 8·5 | 0·14 | 0·07 | 0·28 |
| 4 | | 1,470 | 7·6 | 0·13 | 0·05 | 0·31 |
| 5 | | 1,780 | 9·9 | 0·15 | 0·08 | 0·44 |
| 6 | 1·3 gm. alanine | 1,130 | 7·3 | 0·14 | 0·05 | 0·31 |
| 7 | | 920 | 8·1 | 0·10 | 0·07 | 0·35 |
| 8 | | 560 | 7·2 | 0·07 | 0·04 | 0·30 |
| 9 | 1·25 gm. methionine | 900 | 8·7 | 0·11 | 0·07 | 0·55 |
| 10 | | 1,000 | 8·9 | 0·12 | 0·08 | 0·54 |
| 11 | | 750 | 7·5 | 0·09 | 0·07 | 0·27 |
| 12 | 1·3 gm. glycine | 840 | 8·1 | 0·09 | 0·06 | 0·41 |
| 13 | | 800 | 9·8 | 0·10 | 0·08 | 0·49 |
| 14 | | 750 | 6·7 | 0·10 | 0·06 | 0·26 |
| 15 | 2·2 gm. leucine | 1,100 | 6·3 | 0·13 | 0·06 | 0·24 |

*Homocystine.* If, as recent evidence suggests, the main path in the breakdown of methionine to cystine involves its demethylation to homocysteine, then it would be expected that the latter (and homocystine) would increase cystinuria. Homocysteine has not, however, been isolated from tissues, and therefore may not be a true intermediate though its metabolic breakdown may follow the same path as that of methionine.

In the present case, after the ingestion of 1·1 gm. homocystine, there was a rise in urinary sulphate, cystine and methionine which would account for 15, 17 and 24 p.c. respectively of the ingested amino acid although the concurrent nitrogen excretion fell from 8·8 to 7·4 gm. per day. This appears to be the first record of extra cystine being excreted after the ingestion of homocystine. Lough *et al.*, (1941), obtained no increase in cystine excretion when homocystine was given to a cystinuric patient who was consuming a high protein diet, but under these conditions methionine also was ineffective. Brand, Cahill and Block (1935), recovered about 32 p.c. ingested homocystine as sulphate and 24 p.c. unchanged but none as cystine. However, when homocysteine was given to their patient both cystine and sulphate excretion rose. They also state (cf. Butz and du Vigneaud, 1932) that the Lugg-Sullivan modification for cystine determination (which was used in the present experiment) was not affected by homocystine, so that the apparent rise in cystine excretion in the present case cannot be attributed to unchanged homocystine.

Unfortunately only a little homocystine was available and, although a small amount did not appear to affect the methionine titration, there was insufficient material to confirm these results. However, as, under the conditions of the titration, neither cystine nor cysteine are oxidized it seems unlikely that homocystine would interfere with the estimation. Nevertheless, it should be borne in mind that it is possible that some of the estimated methionine may really be due to homocystine.

TABLE 2.

*Extra sulphur excreted after various amino acids.*

| Amino acid supplement. | Date. | Average N (gm.) | Extra sulphur excreted as Cystine gm. | p.c. | Methionine gm. | p.c. | Sulphate gm. | p.c. | P.c. S-containing amino acid recovered. |
|---|---|---|---|---|---|---|---|---|---|
| 1 gm. Cystine | April 27–28 | 10·2 | 0 | 0 | 0·02 | 7 | 0·40 | 148 | 155 |
| 1·25 gm. Methionine | May 9–10 | 8·8 | 0·08 | 30 | 0·06 | 24 | 0·50 | 180 | 234 |
| 1·1 gm. Homocystine | April 30–May 1 | 6·8 | 0·05 | 17 | 0·06 | 24 | 0·04 | 15 | 56 |
| 1·3 gm. Serine | May 3–4 | 8·0 | 0·04 | | 0·01 | | —0·05 | | |
| 1·3 gm. Alanine | May 6–7 | 7·7 | 0·01 | | 0 | | —0·07 | | |
| 1·3 gm. Glycine | May 12–13 | 9·0 | 0 | | 0 | | 0·35 | | |
| 2·2 gm. Leucine | May 15 | 6·3 | 0·03 | | 0 | | 0·02 | | |
| 5 gm. Methionine + 3·5 gm. Serine | April 2–4 | 10·0 | 0·17 | 16 | 0·15 | 14 | 0·63 | 59 | 90 |
| 5 gm. Methionine + 4·4 gm. Leucine | March 28–30 | 8·0 | 0·25 | 23 | 0·14 | 13 | 0·64 | 59 | 95 |
| 5 gm. Methionine + 3·0 gm. Alanine | April 7–9 | 9·5 | 0·09 | 8 | 0·08 | 7 | 0·74 | 78 | 93 |
| 5 gm. Methionine + 2·5 gm. Glycine | April 12–14 | 8·8 | 0·18 | 17 | 0·11 | 10 | 0·74 | 78 | 105 |

If the method for determining methionine does not include homocystine then the increased excretion following the ingestion of homocystine could be attributed to the synthesis of methionine from homocystine; Bennett and Toennies (1946) and du Vigneaud *et al.* (1939), have found that under suitable circumstances rats can utilize homocystine as a source of methionine. Another explanation is that homocysteine and methionine compete for enzymes and substrates responsible for their oxidation to cysteine, so that methionine oxidation is inhibited. Similarly, rate of breakdown of methionine by this means may be limited when the intake of methionine is high and cysteine production from added methionine would then be restricted. Some other mechanism would then be responsible for the conversion of methionine to the extra sulphate obtained at high protein levels. In this respect it is of interest to note that although equivalent amounts of homocystine and methionine caused similar increases in cystine excretion much more extra sulphate was obtained from methionine.

*2. The Effect of Single Small Doses of Sulphur Free Amino Acids.*

*Serine.* Binkley and du Vigneaud (1942) and Stetten (1942) have presented evidence to show that the following reaction can occur in tissues: homocysteine + serine ⟶ cysteine. It is therefore conceivable that an inadequate serine intake might be a limiting factor in the production of cysteine.

On the other hand Binkley (1943) and Lawrence and Smythe (1943) have shown that in some animals the enzyme which converts cysteine to pyruvic acid resembles and may be identical with that which breaks down serine and that its action is inhibited by serine. If this is so then a high serine intake might limit the destruction of cysteine and so be responsible for increased cystinuria.

When 1·3 gm. serine was given to this cystinuric there was a small rise in cystine excretion and a corresponding fall in sulphate excretion, while the urinary methionine and nitrogen were scarcely affected. The extra cystine was nearly as great as after an equivalent

amount of homocystine but, as already explained, the effect of the latter has probably been underestimated and the real difference should be greater. An examination of Fig. 1, where the sulphur excretion is related to urinary nitrogen, gives a better indication of the relative effects of these two amino acids.

Serine, then, did cause a small rise in cystine excretion at the expense of the urinary sulphate.

*Alanine.* Hess and Sullivan (1942), found that alanine increased the cystine excretion in one of two cystinurics studied. Cleland (1946), in another cystinuric, also found that extra cystine was obtained after taking alanine and suggested that it might be due to a reversal of the reaction which is responsible for the formation of alanine in tissue slices during the production of $H_2S$ from cysteine (though neither Fromageot (1940), nor Lawrence and Smythe (1943) found any inhibition *in vitro*). Another tentative explanation was that alanine might be a source of serine which might be a limiting factor in cysteine formation.

In the present case, when 1·3 gm. alanine was given, there was a fall in urinary nitrogen corresponding to a reduction in protein catabolism during that period, and this reduction may have masked any absolute increase in cystine excretion caused by the alanine. There was, however, a very definite rise in the ratio of cystine excreted per gm. of nitrogen, and as this was not accompanied by a corresponding increase in the relative excretion of sulphate and methionine, it suggests that in this case, too, alanine caused an increase in cystine excretion.

*Glycine.* Because methionine is utilized for its methylation, glycine might be expected to influence methionine metabolism.

Luck and Amsden (1931), reported that, in dogs, parenteral administration of amino acids including glycine and alanine stimulated both protein catabolism and sulphate excretion, while Reid (1939), obtained a marked increase in urinary sulphur and nitrogen but a fall in the ratio of sulphate to nitrogen excretion when dogs were given large doses of glycine and alanine, (25 and 30 gm. respectively).

Hess and Sullivan (1942), found that glycine increased cystine excretion in the same cystinuric who responded to alanine. This was accompanied by a rise in urinary sulphate and may have been due to increased endogenous metabolism rather than to a specific action on cystine. No increase in cystinuria after the ingestion of 5 gm. glycine was obtained from the other cystinuric studied by me (Cleland, 1946) and the marked rise in urinary sulphate was accompanied by an equivalent increase in nitrogen excretion. On the other hand, in the earlier experiments on the present patient (Cleland, 1945), when gelatine (which contains large amounts of glycine) was substituted for other proteins in the diet the cystine, methionine and in particular the sulphate excretions were greater than would have been expected on theoretical grounds, and, on the medium protein diet (N excretion = 7·8 gm./day), the ratio of urinary sulphate to nitrogen was increased. In the present experiment, after a single small dose of glycine (1·3 gm.), cystine and methionine excretion were unaltered but there was a rise in the ratio of urinary sulphate to nitrogen which was not, however, as great as after smaller doses of the S-containing amino acids. In this individual, then, glycine not only increased the endogenous protein metabolism but also, and to a relatively greater extent, stimulated sulphate excretion. This apparently specific action on urinary sulphate may be merely a reflection of the type of protein being catabolized (e.g. those rich in cystine such as serum proteins), rather than a specific action on methionine metabolism, as there was not a corresponding fall in cystine and methionine excretion.

*Leucine.* As leucine is biologically rather inert and has not yet been shown to be involved in the metabolism of any of the S-containing amino acids, it was chosen as a suitable control for these experiments. It was, therefore, surprising to find that the ingestion of 2·2 gm. leucine was followed by a relative and an absolute rise in cystine excretion while methionine and sulphate excretion were practically unaltered. Both on this occasion and when leucine was given with methionine the nitrogen excretion was unusually low indicating that less protein was being catabolized. In addition these results include only extra sulphur excreted during the day on which the amino acid was fed. The value given for extra sulphur excreted as cystine is therefore too low. If these results are to be compared with those obtained after other amino acids and in which concurrent nitrogen excretion was appreciably higher, then it should be remembered that the value for sulphur excretion per gm. of nitrogen will tend to be high when nitrogen excretion is unusually low.

Unfortunately, owing to the onset of menstruation specimens of urine were not collected on the following day and menstruation itself may have affected the results.

*3. The Effect of Sulphur-Free Amino Acids in Conjunction with 2·5 gm. Methionine.*

*Methionine and Leucine.* Although leucine was used as a control amino acid a single small dose was found to increase cystine excretion, and when it was given in conjunction with methionine the p.c. of the latter recovered as cystine (23 p.c.) or excreted per gm. of N was greater than after the other amino acids tried. The excretion of sulphate S per gm. of N was also higher than after other test doses of the amino acids although this accounted for only 60 p.c. methionine; but, as already explained, the absolute S excretion is probably too low and the

relative excretion too high. Concurrent methionine excretion was not affected by the addition of leucine to a test dose of methionine.

*Methionine and Serine.* The possibility that the variations in the p.c. recovery of methionine at different levels of protein intake might be due in part to inadequate supplies of serine has already been discussed (Cleland, 1945 and 1946), and it was thought that the recovery of extra cystine from a test dose of methionine might be increased if additional serine were available at the same time. After taking a total of 5 gm. methionine and 3·5 gm. serine the extra sulphur recovered as cystine, methionine and sulphate amounted to 16, 14, and 59 p.c. respectively, values which, owing to a concurrent rise in nitrogen excretion, are probably too high. When compared with the results obtained after leucine and methionine, there was an almost identical recovery of extra sulphate, a trace more methionine and appreciably less cystine when serine was used. These differences are of the same order as those obtained when small amounts of serine or leucine alone were given. Though it is possible that some of the extra cystine was due to the effect of the serine this could not have been very great and so lack of serine cannot be considered as a major factor limiting the production of cystine from added methionine.

*Methionine and Alanine.* As with leucine, a small dose of alanine was found to increase the urinary cystine without affecting the sulphate and methionine excretion. Yet when alanine was given with methionine the excretion of extra cystine and methionine and the increase in urinary cystine and methionine excreted per gm. of nitrogen were lower than when the other amino acids were given with the methionine. The excretion of extra sulphate was rather high (78 p.c. ingested methionine) and the total extra urinary sulphur accounted for 93 p.c. of the methionine. Why alanine should restrict the excretion of cystine from methionine when by itself it increases the urinary cystine is difficult to decide. Perhaps in the presence of excess alanine the methionine undergoes some different metabolic reaction or the alanine inhibits one of the steps in its usual breakdown to cysteine. Neither of these possibilities seems particularly satisfactory. Its action on methionine metabolism was similar to that of a high protein diet, and possibly the same factors may be responsible.

*Methionine and Glycine.* Glycine alone was found to increase the sulphate excretion, but not urinary cystine or methionine. In conjunction with methionine the sulphate excretion was high (78 p.c. methionine) and the extra cystine (16·8 p.c.) was of the same order as after serine and methionine, and the methionine (10 p.c.) somewhat lower, though not as low as after alanine and methionine. When these results are expressed as cystine, methionine and sulphate sulphur excreted per gm. of nitrogen the values resemble those obtained after equivalent amounts of leucine and methionine. The total sulphur recovered was greater than could be accounted for by the methionine and the excess was probably due to the stimulation of sulphate excretion by the glycine.

Glycine, then, did not affect appreciably the excretion of the sulphur-containing amino acids derived from added methionine, but urinary sulphate was very much greater.

## SUMMARY.

Total nitrogen and cystine, methionine and sulphate sulphur have been determined in the urine of a cystinuric woman.

Ingested cystine increased urinary sulphate but did not affect the excretion of S-containing amino acids.

Both methionine and homocystine when taken by mouth increased the excretion of cystine, methionine and sulphate sulphur.

Single small doses of serine, alanine and leucine did not alter methionine excretion but increased urinary cystine and in the case of serine reduced the urinary sulphate.

Glycine taken by mouth caused a marked rise in urinary sulphate but the excretion of the sulphur-containing amino acids was unaltered.

Recovery of unchanged methionine after a test dose was greatest (14 p.c.) if leucine or serine were given in addition, and least (7 p.c.) when alanine was added. After adding an equivalent amount of glycine, 10 p.c. of the methionine was recovered unchanged.

The recovery of extra cystine from added methionine was greatest (23 p.c.) when leucine was given as a supplement, and least (8 p.c.) when alanine was added. When the supplement was serine or glycine, 16 and 17 p.c. respectively of the methionine could be accounted for as extra cystine.

Extra sulphate after the test dose of methionine was 59 p.c. when serine or leucine were given in addition, and rose to 78 p.c. when equivalent amounts of alanine or glycine were used.

Possible interpretations of these results are discussed.

REFERENCES.

Bennett, M. A. and Toennies, G. (1946): J. biol. Chem., 163, p. 235.
Binkley, F. (1943): *Ibid.*, 150, p. 261.
Binkley, F. and du Vigneaud, V. (1942): *Ibid.*, 144, p. 507.
Brand, E., Cahill, G. F. and Block, R. J. (1935): *Ibid.*, 110, p. 399.
Butz, L. W. and du Vigneaud, V. (1932): *Ibid.*, 99, p. 135.
Cleland, J. B. (1945): Austral. J. exp. Biol., 23, p. 273.
Cleland, J. B. (1946): *Ibid.*, 24, p. 219.
Fromageot, C., Wookey, F. and Chaix, P. (1940): Enzymologia, 9, p. 198.
Hess, W. C. and Sullivan, M. X. (1942): J. biol. Chem., 42, p. 3.
Lawrence, J. M. and Smythe, C. V. (1943): Arch. Biochem., 2, p. 225.
Lewis, H. B., Brown, B. H. and White, F. R. (1936): J. biol. Chem., 114, p. 171.
Lough, S. A., Perilstein, W. L., Heinen, H. J. and Carter, L. W. (1941): *Ibid.*, 139, p. 487.
Luck, J. M. and Amsden, M. R. (1931): Proc. Am. Soc. biol. Chem., J. biol. Chem., 92, p. lxv.
Reid, C. (1939): Biochem. J., 33, p. 723.
Stetten, de W. (1942): J. biol. Chem., 144, p. 501.
du Vigneaud, V., Chandler, J. P., Moyer, A. W. and Keppel, D. M. (1939): *Ibid.*, 131, p. 57.

# THE ACCUMULATION OF VITAMIN C DURING GERMINATION IN PEAS

by T. S. BRAILSFORD ROBERTSON

(From the Department of Biochemistry, University of Adelaide).

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It has been known for a considerable time that germination conferred antiscorbutic properties on certain seeds. Harris and Ray (1933) showed that hexuronic acid appeared concurrently with antiscorbutic properties in germinating peas. They used for their estimations of the acid a simple indophenol titration method, and in that way only obtained figures for the accumulation of "ascorbic acid." Theirs was the first experiment to show the accumulation of the vitamin on consecutive days. v. Hausen (1936) has shown that pea seedlings which have lost their cotyledons after a period of seven days' germination show the hormonal effect of the vitamin added to the culture media, whereas those which have no vitamin C added rapidly die. Bonner and Bonner (1938) showed that in the culture of plant embryos the vitamin C content rises to a maximum within two weeks, and remains at this level for the following two weeks. Povolockaja (1937) gives figures for estimations of ascorbic acid in samples of germinating whole peas of ages of 1, 3, 5, 9, and 12 days. She also states that in loosing their germinating power fully or partially, the seeds lose to the same extent their ability to form vitamin C. She states that absolutely non-sprouting seeds fail to show a trace of vitamin formation, and that the cotyledons of the Leguminosae, even if deprived of their radicle, can form vitamin C independently, while the endosperm of the cereals is devoid of this power. She finally states that "the fact that the seeds of the Leguminosae take one to three days to form large quantities of vitamin C is apparently due to the ability of the cotyledons of the Leguminosae to synthesize the vitamin." She gives a value of 97·2 mg. ascorbic acid/100 gm. for a pea which had evidently had its embryo removed before germination had begun, and 40·6 mg./100 gm. of original air dry weight for an entire germinating pea. She defines no conditions under which the germination was carried out. Lugg (1943) gives a series of figures for vitamin accumulation in Blue Boiler peas. The curves drawn from the foregoing references are shown in Fig. 1.

The present investigation was begun before the reference was found to Povolockaja's paper, and was intended to ascertain the effect of the presence of the embryo on processes occurring in the cotyledon. Some of the results of Povolockaja have been confirmed and hitherto unpublished data on the cause and course of accumulation of vitamin C are presented.

## EXPERIMENTAL METHODS.

*Ascorbic acid.* Four experiments were carried out (I, II, III and IV) in which the accumulation of ascorbic acid and dehydroascorbic acid in peas from which the embryo had been removed ("treated peas") was compared with that in whole (control) peas. In experiments I and II, Blue Boiler peas were used, being three years old, and showing a viability of 95 p.c. In experiments III and IV, variety "Dunn," 9–15 months old showing 90–95 p.c. germination were used. In all cases, the peas were hand picked before germination, and in appropriate batches the embryos were removed by cutting away with a scalpel, or by using a small dentist's drill. Treated and control lots were sterilized by immersion in dilute $HgCl_2$ solution for 15 minutes, and finally washed for 5–10 minutes in a stream of glass-distilled water.

All seeds were soaked in glass-distilled water for 18 hours, and planted out on filter papers or cotton-wool kept moist with glass-distilled water. Peas which had not germinated after 72 hours were discarded, and their dry weight subtracted from the batch weight. Details of variations from the above proceedure are set out for individual experiments below:

Expt. I. Temperature 16° C. in natural daylight.
Expt. II. Temperature 25° C. in natural daylight.
Expt. III. Temperature 24–29° C. in diffuse daylight.
Expt. IV. Temperature 30° C. constant under 800 watt lamp placed 2 feet above the bench.

In Experiment IV, duplicate batches of treated and control peas were germinated in complete darkness, and in addition, 2 series of batches of powdered peas were included, the latter being dampened and treated as all other batches.

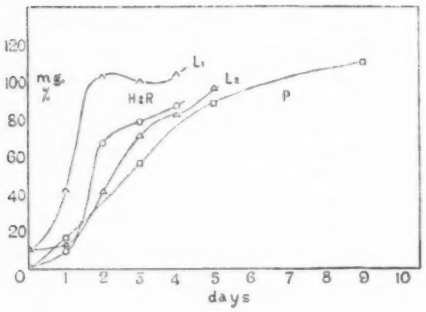


**Fig. 1.** L1, Green Gram (Lugg, 1943); L2, Blue Boiler (Lugg, 1943); H & R, Harris and Ray (ascorbic acid only); P, Povolockaja (1937).

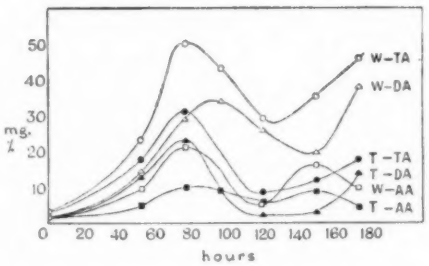


**Fig. 2.** Results of accumulation of ascorbic, dehydroascorbic, and total ascorbic acids on successive days in Expt. III.

Lugg's modification (1942) of the dichlorphenol-indophenol titration was used after homogenising the peas in a Waring Blendor in 6 p.c. $HPO_3$.

*Ascorbic acid oxidase.* Peas were treated and germinated under the same conditions as in Experiment IV.

Method. Each day a batch of peas was homogenised in the Waring Blendor in 150 ml. of glass-distilled water at the maximum speed for 90 seconds. Combined washings took the final volume to 200 ml. From the final suspension of pea tissue, a tenth aliquot (20 ml.) was placed in a 100 ml. stoppered Erlenmeyer flask. To this were added 10 ml. of an 0·2 p.c. ascorbic acid solution. The mixture was immediately shaken, and from it 2 ml. were taken in a 100 ml. beaker, 2 ml. of 16 p.c. $HPO_3$ added, and the resultant mixture titrated against an 0·2 p.c. solution of 2:6 dichlorphenol-indophenol. In addition to this titration carried out at the instant after the addition of ascorbic acid, similar titrations were done at times approximately 30, 60, 90, etc., minutes after the first estimation, and the results are plotted in comparative rates of decolourisation of standard dye.

## RESULTS.

Results in all experiments are expressed as mg./100 gm. air-dry weight. Figures for average "ascorbic acid" accumulation in Experiments I and II in treated and control peas were as follows:

Expt. I. Control peas 72 hours old 16·7 mg./100 gm.
Expt. I. Treated peas 72 hours old 14·5 mg./100 gm.
Expt. II. Control peas 72 hours old 15·3 mg./100 gm.
Expt. II. Treated peas 72 hours old 29·7 mg./100 gm.

Peas before treatment or soaking were found to contain 1·4 mg./100 gm. ascorbic acid, 1·4 mg./100 gm. dehydroascorbic acid, and 2·8 mg. per 100 gm. total ascorbic acid.

Results are given in the form of Figs. 2–9.

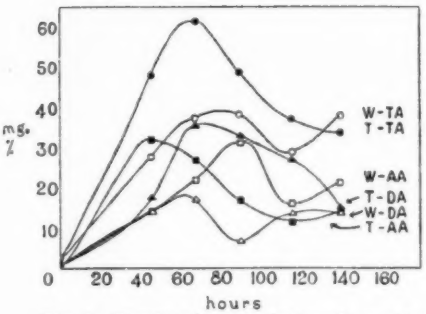


**Fig. 3.** Results of accumulation of ascorbic acids on successive days in Expt. IV.

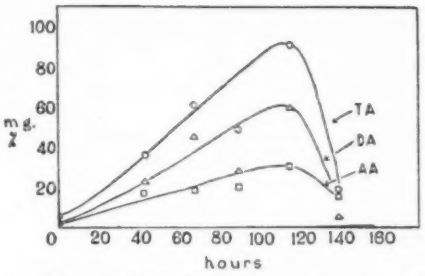


**Fig. 4.** Expt. IV whole peas grown in darkness.

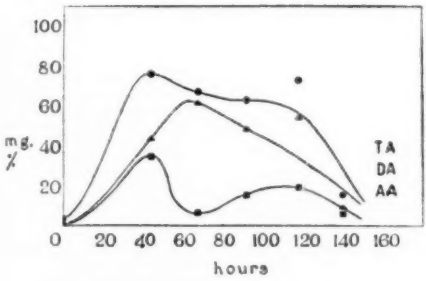


**Fig. 5.** Expt. IV treated peas grown in darkness.

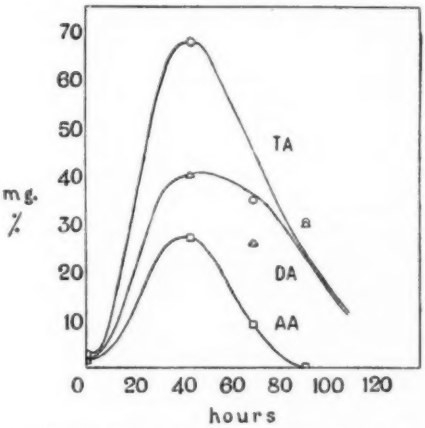


**Fig. 6.** Expt. IV vitamin accumulation in pea powder.

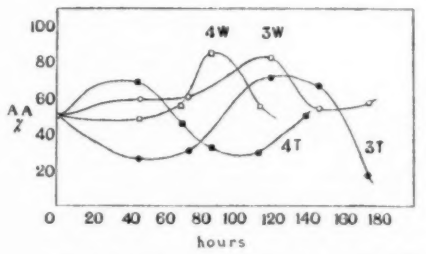


**Fig. 7.** Ascorbic acid expressed as a percentage of total ascorbic acid in Expts. III and IV.

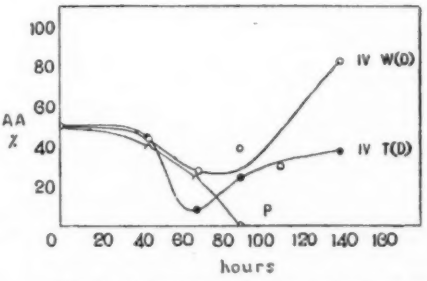


**Fig. 8.** Ascorbic acid as a percentage of total in peas grown in darkness, and in pea powder.

**Notation.** Except on Fig. 1, notation on graphs is as follows: W. whole pea; T, treated pea; AA, ascorbic acid; D.A., dehydroascorbic acid; T.A., total ascorbic acid; 3, Expt. III; 4, Expt. IV; P, pea powder; D, growth in darkness. Numbers on Fig. 9 refer to the hour at which the batch was harvested.

## DISCUSSION.

The fact that a pea in which the embryo has been removed can still produce ascorbic acid which was first noted by Povolockaja is thus confirmed; and the fact that a pea powder, moistened and treated in the normal way will produce high concentrations of the vitamin would show that the processes responsible for the synthesis are inherent in the cell of the cotyledon, and are in no way dependent on the growth of the embryo above it.

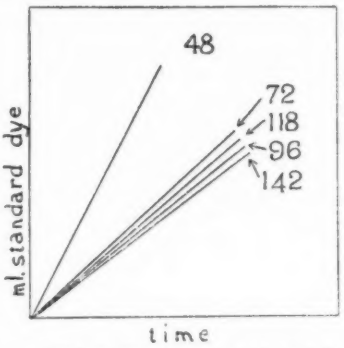


**Fig. 9.** Comparative rates of decolourisation of dye by "ascorbic acid oxidase" produced on consecutive days.

*The Period from 0–70 Hours.*

As regards the absolute amounts of total ascorbic acids, at 72 hours after soaking, whole peas in Expts. III and IV had higher ascorbic acid contents than those in Expts. I and II. Treated peas in II and IV had higher contents than those of I and III. In Expts. II and IV, the temperature was higher than in the latter pair. Light differences would appear to affect the results. The interaction of different factors is important—light, heat, moisture, as these factors also directly affect growth processes which may in turn affect, or be governed by, the vitamin concentration.

v. Hausen (1936) has shown that on administration of vitamin C to culture fluids containing germinating seeds, the content of vitamin C in seeds is increased above the normal quantity synthesized to a definite maximum for each type of pea. She suggests that there is apparently in these plants a mechanism for maintaining the maximum, whereby the excess vitamin C is either inactivated, or transformed into other compounds. Davis *et al.* (1937) have shown that although ascorbic acid added to culture media containing isolated embryos stimulated their development and growth, higher concentrations retard their development, and are prejudicial to the life of the plants.

In Expt. III, conditions appeared better for growth of root and shoot, which was rapid and strong. From Fig. 2, it would appear that the cotyledon builds up a steady reserve of ascorbic acid, which reserve, in the whole pea, may be drawn upon for the use of cells in the descending or ascending axes, thus leaving a deficit in the cotyledon, which is continuously being made good. In this way, the amount of total ascorbic acid in the whole plant would represent the amount synthesized in the cotyledon, plus the amount held in the ascending and descending axes. In Exp. IV, at higher temperatures, and better light conditions, concentrations are capable of being built up in the cotyledons, but these concentrations are not seen in the intact plant germinating under identical conditions.

*The Period from 70–120 Hours.*

Over this time, a pronounced fall in the concentration of total ascorbic acid is seen in all curves, and both in whole and treated peas. The fall in the curves for treated peas can be attributed to the fact that after 60–80 hours at these moderately high temperatures, available carbohydrate reserves are probably exhausted, and production of the vitamin ceases. Other processes as yet unexplored, but which also may require the vitamin, may reduce the concentration after this time. However, in the curve of synthesis of the vitamin in moistened pea powder, it will be seen that the vitamin C production ceases after a very short time—in this case 100 hours. This has invariably been found to be the case—that the production in the cotyledon cells would appear to follow a definite temperature-time-particle size relationship; that is to say high temperatures and small particle size lead to greater production of ascorbic acid in less time, while a decrease in temperature, or an increase in the size of the particle spreads the accumulation over a greater period, and exceptionally high concentrations are not reached. This general rule is of course limited by the optimum temperature above which the enzyme reactions are inhibited to a greater and greater extent until they are brought to a stop, and also by the size of the cotyledon cell, below which dimension the vitamin may not be synthesized; intact cells, in the greatest proportion possible, would seem to be necessary.

In the whole pea, however, there would seem to be complications. The peaks of the curves in Expts. III and IV coincide with the emergence of the ascending axis. The drop in the curve in the intact pea may be attributed not only to a cessation of production in the cotyledons, but also to an increased utilization in the ascending axis (Reid, 1941). For this reason, the curve in production in whole peas should drop away more steeply than that in treated peas,

there being no growth in the latter. This phenomenon is, however, not seen. In any case, the proportion of new tissue formed in the axes compared with the bulk of the cotyledon is small, and added to this, the treated peas have cut and exposed tissues unprotected by any testa, and some of the vitamin produced is almost certainly leached from the surface cells, and some may also be oxidized at the cut exposed surfaces. Thus the difference in the rate of fall of the two curves, if it exists, would perhaps not be apparent.

*Shape of the Curve.*

The shape of the various curves has been consistent throughout the experimental work, and suggestions must be made as to why this characteristic curve has not evidently been noted previously.

Low temperatures are conducive to a slow production of the vitamin in the cotyledons, spread over a longer period of time. Thus, if the appearance of the ascending axis is not delayed unduly, and photosynthesis begins moderately early, the level of ascorbic acids in the cotyledon does not drop until after photosynthesis has begun in the leaves—thus a drop in the curve of accumulation in the whole plant would not be seen.

In all cases, the first appearance of leaves and of green tissue occurred just before the lowest point reached on the graph; in the case of Expt. III at 110 hours, and in Experiment IV at 100 hours. It is presumed that photosynthesis then begins. Providing then that the drop in the curve is due to the cessation of the synthesis of the vitamin in the cotyledon, the subsequent rise in the whole pea is presumed to be due to the formation in the photosynthesising leaves of available carbohydrate, and thus, later, of "total ascorbic acid." Thus the drop in the curve, giving the graph of characteristic shape, may not invariably occur—a flatter curve may be obtained using other varieties of peas at lower temperatures. This is probably the reason that the effect has not hitherto been noticed.

*Growth in the Dark.*

The presence of an etiolated shoot delays the maximum in ascorbic acid production (Figs. 4 and 5). Very high concentrations of "total ascorbic acid" are produced in whole peas, which is probably due to the more humid conditions of their growth and to the fact that as etiolation is a vacuolation effect, producing little actual growth, ascorbic acid is not needed for the elongation of cells by active growth (Reid, 1941) and high concentrations are built up in the cotyledon. In growing peas in the light, darkening of the seed invariably occurs, but in the dark, little damage to the surface cells occurs, and the peas are of a uniform clean white colour. The former condition may predispose to a loss of vitamin C in the seed, while darkness may tend to preserve the surface vitamin C, allowing a higher overall concentration to develop. The fact that there may be a decrease in the utilization of vitamin C in an etiolated pea is further supported by the late maximum point in the curve, and even more by the sudden drop in the vitamin C concentration due to carbohydrate exhaustion. The lack of light in no way affects the accumulation in treated peas, which is in every way normal, though high, probably due to a minimization of surface oxidation and humid conditions.

*Synthesis of the Vitamin.*

The question of whether ascorbic acid or dehydroascorbic acid is primarily involved in carrying out the vitamin function is an interesting one. It would appear from results obtained that in every case in treated peas ascorbic acid was the first to disappear when synthesis of the vitamin ceased in the cotyledon. The most pronounced case of this was that of the pea powder, where ascorbic acid was not demonstrable after 90 hours, although dehydroascorbic acid persisted until 120 hours. Although it is by no means conclusive the results would tend to suggest that the reaction ascorbic acid $\xrightarrow{-2H}$ dehydroascorbic acid is the first and main functional one for the cotyledon cells. While production in the cotyledon is the main factor in ascorbic acid synthesis in the seedling, ascorbic acid itself is almost invariably low, when expressed as a percentage of total (Figs. 7 and 8). Later, however, in the whole pea, when synthesis in the leaf is the main factor, even although the turnover of the vitamin is possibly higher than it was before, ascorbic acid itself increases on the average to 80 p.c. of the total, which may be due either to a reversal of the reaction in this part, or a decrease in the ratio of utilization/synthesis, which is unlikely at that stage of growth.

*Ascorbic Acid Oxidase.*

Determinations of oxidase done on whole peas showed no marked change from day to day. There was a tendency, however, for high concentrations during the first 48 hours, and then a progressive decrease until after the fifth day. In that way the concentrations of ascorbic acid oxidase would tend to follow the ascorbic acid concentration. It would appear that other systems must be investigated besides the ascorbic acid oxidase if a true explanation for the fluctuations of the ascorbic acid concentrations is to be found.

SUMMARY.

Certain pecularities in the graphs of synthesis of vitamin C in germinating peas are discussed.

It is suggested that temperature, and later light, play a large rôle in determining the nature of the course of vitamin C synthesis.

Peas whose embryos have been excised before germination (treated peas) will, if soaked and planted under laboratory conditions, produce high concentrations of vitamin C, in some conditions, even higher concentrations than those obtained in an intact pea under the same conditions.

The synthesis of vitamin C is shown to occur in the cotyledon in any cell or group of cells, regardless of the degree of subdivision of the cotyledon.

Light is found not to be necessary for the synthesis of vitamin C in the cotyledon, but to be necessary before synthesis in the leaf can occur.

It would appear that the variation of the concentration of total ascorbic acid cannot be wholly attributed to variations in concentration of the ascorbic acid oxidase, which remains constant after the first two days.

It is suggested that the production of too high concentrations of vitamin C for the optimum growth of the seedling may be prevented by a limiting factor within the intact pea.

It is suggested that the vitamin is produced in the form of "ascorbic acid," and that the function of the vitamin in the cotyledon of the seedling can be expressed by the equation: ascorbic acid $\xrightarrow{-2H}$ dehydroascorbic acid.

*Acknowledgments.* My thanks are due to Dr. J. W. H. Lugg, who offered much helpful advice, encouragement, and criticism throughout the duration of this work; and to Mr. J. A. O'Brien, and others, who helped in the preparation of materials for the execution of these experiments.

REFERENCES.

Bonner, J. and Bonner, D. (1938): Proc. Natl. Acad. Sci. U.S., 24. p. 70.
Davis, W., Atkins, G. A. and Hudson, P. C. B. (1937): Ann. Bot., 1, p. 329.
Harris, P. L. and Ray, S. N. (1933): Biochem. J., 27, p. 580.
Lugg, J. W. H. (1942): Austral. J. exp. Biol. 20, p. 274.
Lugg, J. W. H. (1943): *Ibid.*, 21, p. 111.
Povolockaja, K. L. (1937): C. R. Acad. Sci. U.R.S.S., 18, Nos. 1 and 2.
Reid, M. E. (1941): Amer. Bot., 28, p. 410.
v. Hausen, S. (1936): Biochem. Z., 288, p. 378.

# THE RELATION BETWEEN SOME REVERSIBLE REACTIONS OF HAEMOGLOBIN AND THE FORMATION OF GREEN DERIVATIVES.

by HENRY FRANCIS HOLDEN[1]

(From the Walter and Eliza Hall Institute, Melbourne).

*(Accepted for publication 8th October, 1946.)*

For some years it has been known that, in addition to denaturation by heat and other agents which they suffer in common with other native proteins, haemoglobin and some of its derivatives can undergo reversible reactions characterized by temporary changes in spectrum or, in some cases, in solubility. By some authors these changes have been regarded as evidence of a genuine denaturation of the protein and its subsequent renaturation, or perhaps it should rather be said that the classical definition of denaturation has been expanded to include this relatively recently observed class of phenomena. As an example of the accommodating type of definition now in vogue in this field one may perhaps quote: "Denaturation may be defined as some intra-molecular re-arrangement of the protein giving rise to definite changes in the chemical, physical and biological properties" (Anson, 1938). In quoting this definition, Neurath, Cooper and Erickson (1942) carefully add a footnote of their own categorically excluding from "intra-molecular re-arrangement" "the reversible splitting of native proteins into smaller units, or reactions with groups on the surface of the molecules which leave their internal structure intact." If "intact" is intended to mean "unchanged," it would seem that the footnote excludes very little since, e.g. it is almost certain that the conversion of methaemoglobin to oxyhaemoglobin profoundly alters the internal structure of the globin molecule, just as it does some of its physical and chemical properties.

To include in one category these reversible reactions almost peculiar to the blood pigment and those occurring when, say, egg albumin is dropped into alcohol may perhaps be not unjustly likened to the use of one phrase to include both a gentle squeeze of a tennis ball between the fingers and the turning of the said ball, after suitable dissection, inside out. Nor does it seem easy to align the fact that denaturation, as usually understood, consists in the rupture of a large number of weak bonds in a native protein of almost unique constitution, thus transforming it into one of exceedingly random structure (Mirksy and Pauling, 1936) with the extraordinary ease with which some reactions termed denaturation have been reversed. There is another class of reactions of the blood pigment by means of which are produced green pigments possessing in the ferrous state a strong absorption band in the red region of the spectrum. Most of these pigments have not yet been obtained in a pure state, nor have their constitutions and relationships been fully established. The common feature in the reactions appears to be the action of an oxidizing agent on one of the ferrous derivatives of the blood pigment. A comparative study of the reversible changes that the blood pigment can endure and of the preparation of green pigments has extended the list of each type of reaction and suggests that they may be more closely connected than would at first seem likely.

[1]This work was aided by a grant from the Australian National Health and Medical Research Council.

Whatever one's view of protein denaturation, it is helpful to distinguish accurately between different types of reactions of proteins; the practice in this paper follows that of previous ones in this series of reserving the term denaturation for examples of those reactions whose resultants come within the classical definition applicable to denatured proteins from any source: *i.e.* that they can be repeatedly precipitated at their iso-electric points but are soluble away from them. As a simple term for reversible reactions of the blood pigment whose resultants are readily convertible to it the word "perturbation" will be used and the word "restoration" for the reconversion of such a resultant to a protein soluble at its iso-electric point and containing unaltered haem or haematin. As yet the occurrence of analogous reactions with other native proteins is, perhaps, a matter for conjecture rather than for affirmation.

## EXPERIMENTAL.

The experiments to be described fall into three main groups: 1, a study of the effects on haemoglobin and methaemoglobin of certain reagents known to denature oxyhaemoglobin; 2, the preparation from haemoglobin of various green pigments with absorption bands in the red region of the spectrum; and 3, the separation from the protein of the prosthetic groups of a few of these green pigments.

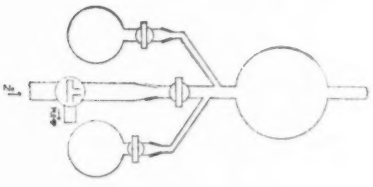

Fig. 1.

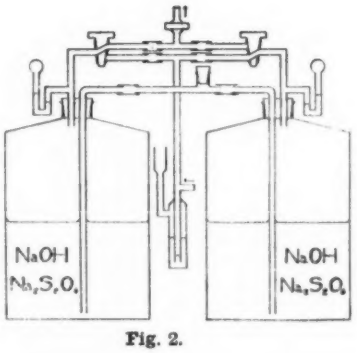


Fig. 2.

The results of many diverse experiments have demonstrated that reducing agents, particularly sodium hyposulphite, have an action on oxyhaemoglobin very different from that of an indifferent gas or a reduction in the gaseous pressure in desoxygenating it. Accordingly the studies on haemoglobin were again pursued in an apparatus similar to that previously used (Holden, 1937) for this purpose. The rubber tubing was replaced by ground joints and the screw clips by taps. The apparatus was diminished to a total volume of 150 ml. and to the main bulb was added a tube in which observations on a film of liquid could be made with a Hartridge spectrometer supported vertically. (Fig. 1). The apparatus was mounted on a board that was rotated at about 100 r.p.m. by a motor. The only change in the apparatus for supplying nitrogen freed from oxygen was a corresponding replacement, as far as possible, of rubber by glass and clips by taps. (Fig. 2). The solutions were placed in the three flasks with capillary pipettes; the technique was otherwise unchanged. To ensure that experiments on methaemoglobin should not be vitiated by undetected leakage of air a small amount of oxyhaemoglobin was added to the harmless restorative solution. Where it was desired to saturate the haemoglobin solution with a reagent a moderate excess of the latter in the solid state was placed in the largest flask and the haemoglobin occupied the small one usually reserved for the perturbator.

As solutions of haemoglobin and oxyhaemoglobin washed and laked erythrocytes were used. Methaemoglobin was prepared by autoxidation of oxyhaemoglobin at 37° C. and pH 5·3. Its freedom from undesirable reagents such as traces of potassium ferricyanide outweighed the disadvantage of its including a small percentage of native protein joined to an altered prosthetic group. Experiments showed that crystallized horse cyanmethaemoglobin prepared by autoxidation had a maximum visible extinction coefficient 5–7 p.c. less than that prepared

by the oxidation of oxyhaemoglobin with potassium ferricyanide. It seems, however, very difficult to effect a complete removal of the latter. The methaemoglobin was converted as required to cyanmethaemoglobin with the minimal quantity of potassium cyanide neutralized with acetic acid. The degree of restoration was judged sufficiently closely for present purposes by a comparison of the spectrum of the restored and reduced protein solution with that of a double wedge cell, one compartment of which contained a solution of haemoglobin and the other an equivalent solution of denatured globin haemochromogen. Only where the degree of denaturation was very small was it necessary to separate the denatured protein and determine it separately.

TABLE 1.

A. Substances which denature oxyhaemoglobin but which have little irreversible effect on haemoglobin.

Ammonia.
Ethanol.
Potassium iodide.
Sodium benzoate.
Sodium salicylate.
Sodium benzene sulphonate.

B. Substances which denature any native derivative of haemoglobin.

Phenol.
Pyridine.
Formaldehyde.
Ammonium thiocyanate.
Potassium thiocyanate.

C. Substances which do not denature oxyhaemoglobin but which combine with haemoglobin and promote the formation of green pigments.

Potassium cyanide.

TABLE 2.

*The effect of acid on the derivatives of haemoglobin.*

Sulphuric acid was used because it is not volatile. The period of action was 5 minutes.

A. Derivatives of horse haemoglobin.

| Derivative. | Concn. gm./litre. | Vol. ml. | Gas. | Concn. of acid, N. | Vol. of acid, ml. | pH reached. | Temp. C. | Alkaline restorer. | Concn. of alkali, N. | Vol. of alkali, ml. | p.c. restored. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hb. | 77·5 | 2·5 | $N_2$ | 0·5 | 1·3 | 1·65 | 0° | $Na_2CO_3$ | 1 | 2·5 | 90 | |
| Hb. | 81 | 2·0 | $N_2$ | 0·5 | 2·0 | 1·3 | 0° | $Na_2CO_3$ | 1 | 4·0 | 85 | |
| Hb. | 77·5 | 2·5 | $N_2$ | 0·5 | 1·3 | 1·65 | 20° | $Na_2CO_3$ | 1 | 2·5 | 85 | |
| Hb. | 81 | 2·0 | $N_2$ | 0·5 | 2·0 | 1·3 | 17° | $Na_2CO_3$ | 1 | 4·0 | 80 | |
| $HbO_2$. | 77·5 | 5·0 | Air | 0·5 | 2·6 | 1·65 | 20° | $Na_2CO_3$ | 1 | 5 | Trace. | |
| Methb. | 107 | 1·0 | $N_2$ | 0·05 | 2·0 | 3·84 | 0° | $Na_2CO_3$ | 0·1 | 5 | 100 | Minimal amount of acid to convert to acid haematin. |
| Methb. | 107 | 1·0 | $N_2$ | 0·05 | 2·0 | 3·84 | 16° | $Na_2CO_3$ | 0·1 | 5 | 90 | |
| Methb. | 107 | 1·0 | Air | 0·05 | 2·0 | 3·84 | 0° | $NH_3$ | 0·1 | 2 | 75 | |
| Methb. | 107 | 1·0 | Air | 0·05 | 2·0 | 3·84 | 16° | $NH_3$ | 0·1 | 2 | 50 | |
| CN.Methb. | 30 | 2·0 | Air | 0·05 | 3·0 | 3·6 | 0° | $NH_3$ | 0·1 | 5 | 100 | No acid haematin formed. |
| CN.Methb. | 30 | 2·0 | Air | 0·05 | 4·0 | 2·33 | 0° | $NH_3$ | 0·1 | 5 | Little. | |

B. Derivatives of human haemoglobin.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Hb. | 68 | 2·0 | $N_2$ | 0·05 | 4·0 | 3·34 | 0° | $Na_2CO_3$ | 0·1 | 10 | 100 | Note low acidity sufficient to convert to acid haem. |
| Hb. | 68 | 2·0 | $N_2$ | 0·05 | 4·0 | 3·34 | 16° | $Na_2CO_3$ | 0·1 | 10 | 100 | |
| $HbO_2$. | 69 | 5·0 | Air | 0·5 | 1·0 | 3·3 | 22° | $Na_2CO_3$ | 1 | 2·5 | Little. | |
| Methb. | 137 | 1·0 | $N_2$ | 0·05 | 2·5 | 4·6 | 0° | $Na_2CO_3$ | 0·1 | 5·0 | 100 | Note low acidity sufficient to convert to acid haematin. |
| Methb. | 137 | 1·0 | $N_2$ | 0·05 | 2·5 | 4·6 | 16° | $Na_2CO_3$ | 0·1 | 5·0 | 90 | |
| Methb. | 137 | 1·0 | Air | 0·05 | 2·5 | 4·6 | 0° | $NH_3$ | 0·1 | 3·0 | 90 | |
| Methb. | 137 | 1·0 | Air | 0·05 | 2·5 | 4·6 | 16° | $NH_3$ | 0·1 | 3·0 | 85 | |
| CN.Methb. | 180 | 0·5 | Air | 0·5 | 0·8 | 1·8 | 15° | $NH_3$ | 1 | 1·0 | 50 | Minimum acid to convert to acid haematin. |

The effects of perturbators differ with the temperature of the reaction, the derivative of the blood pigment selected and the species whence it is obtained. For two perturbators, hydrogen ions and ethanol, a comparative study was mode of four derivatives: haemoglobin, oxyhaemoglobin, methaemoglobin and cyanmethaemoglobin of the horse and of man. The last two derivatives were studied both in the presence and in the absence of oxygen. To allow time for a complete admixture of the perturbator with the pigment derivative and a spectrometric observation with the Hartridge while restrieting extraneous effects due to a prolonged exposure to the action of the perturbator, e.g. hydrolysis, a standard time was adopted of five minutes between the addition of the perturbator and that of the restorer. The details of quantities used and the results are in Tables 1–5. Potassium cyanide occupies an apparently unique position. A drop of 15 p.c. horse oxyhaemoglobin in 1 ml. of 30 p.c. potassium cyanide was unaltered. On reduction the spectrum of cyanhaemochromogen with the weak α band was seen which on dilution changed after several minutes to that of haemoglobin. The structure of the first substance cannot be at present defined. It has been established by magnetic measurements that the substance with a very different spectrum formed temporarily when cyanmethaemoglobin is reduced is the true cyanhaemoglobin analogous to carbon monoxide haemoglobin. Unlike other chemicals that were used for perturbation the temporary precipitation near the neutral point usually so characteristic was not observed when potassium cyanide acted on haemoglobin or oxyhaemoglobin. There was only a little salting-out at very high concentrations.

TABLE 3.

*The effect of precipitation with ethanol in neutral solution on derivatives of haemoglobin.*

Unless otherwise stated the restoring agent was 10 ml. water.
The restoring agent was added 5 min. after the ethanol.

A. Derivatives of horse haemoglobin.

| Derivative. | Concn. gm./litre. | Vol. ml. | Gas. | Vol. of ethanol, ml. | Temp. C. | p.c. restored. | Remarks. |
|---|---|---|---|---|---|---|---|
| Hb. | 150 | 1·0 | $N_2$ | 2·0 | 0° | 90 | |
| Hb. | 150 | 1·0 | $N_2$ | 2·0 | 20° | 80 | |
| Methb. | 107 | 1·0 | $N_2$ | 1·5 | 0° | 90 | Ppt. insol. in $H_2O$. Restored with 0·2M $Na_2CO_3$. |
| Methb. | 107 | 1·0 | Air | 1·5 | 0° | 75 | Ppt. insol. in $H_2O$. Restored with 0·2M $Na_2CO_3$. |
| Methb. | 107 | 1·0 | $N_2$ | 1·4 | 19° | 80 | Ppt. insol. in $H_2O$. Restored with 0·1M $Na_2CO_3$ and $Na_2S_2O_4$. |
| Methb. | 107 | 1·0 | Air | 1·0 | 16° | 70 | Ppt. insol. in $H_2O$. Restored with 0·1M $NH_3$ and $Na_2S_2O_4$. |
| CN.Methb. | 106 | 1·0 | $N_2$ | 1·2 | 0° | 100 | |
| CN.Methb. | 106 | 1·0 | Air | 1·2 | 0° | 95 | |
| CN.Methb. | 106 | 1·0 | $N_2$ | 1·2 | 18° | 75 | Ppt. insol. in $H_2O$. Restored with 0·1M $NH_3$. |
| CN.Methb. | 106 | 1·0 | Air | 1·2 | 18° | 65 | Ppt. insol. in $H_2O$. Restored with 0·1M $NH_3$ and $Na_2S_2O_4$. |

B. Derivatives of human haemoglobin.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Hb. | 136 | 1·0 | $N_2$ | 2·0 | 0° | 95 | |
| Hb. | 136 | 1·0 | $N_2$ | 2·0 | 17° | 80 | |
| Methb. | 137 | 1·0 | $N_2$ | 1·1 | 0° | 90 | Ppts. insol in $H_2O$. Restored with 0·1M $NH_3$ and $Na_2S_2O_4$. |
| Methb. | 137 | 1·0 | $N_2$ | 1·1 | 17° | 40 | |
| Methb. | 137 | 1·0 | Air | 1·0 | 0° | 40 | |
| Methb. | 137 | 1·0 | Air | 1·0 | 16° | 0 | Ppt. insol. in $NH_3$. Dissolved with dil. NaOH. |
| CN.Methb. | 180 | 1·0 | $N_2$ | 1·5 | 0° | 65 | |
| CN.Methb. | 180 | 1·0 | $N_2$ | 1·5 | 17° | 50 | |
| CN.Methb. | 180 | 1·0 | Air | 1·0 | 0° | 40 | |
| CN.Methb. | 180 | 1·0 | Air | 1·0 | 15° | 40 | |

The human derivatives are in general more readily denatured than the horse derivatives.

From these experiments the following conclusions may be drawn. There are well-marked differences of species; neither horse nor human blood pigment derivatives are as resistant to denaturation as those of the ox previously investigated. Human derivatives are remarkably easily perturbed by acid in comparison with those of the horse and ox. No record has come to my notice of the haemoglobin of any common animal being converted to acid haem at so low an

acidity as pH 3·3. Human methaemoglobin is even more remarkable, being perturbed and partially denatured at pH 4·6 within five minutes. Experiments on the effect of acid on cyanmethaemoglobin showed that it could withstand a pH that denatured much of the corresponding methaemoglobin. There is, however, no spectral change at that pH and so the results have not been tabulated. To dissociate the hydrogen cyanide from the haematin required an acidity of about pH 2·3 and at that pH denaturation was very extensive. An important point is that methaemoglobin is much more easily denatured (as well as more easily perturbed) than haemoglobin. The difference in stability towards acid between acid haematin derived from methaemoglobin and acid haem from haemoglobin points either to haem being able to stabilize the protein in a way impossible to haematin or to some essential differences within the protein molecules existing before the addition of the acid. Though direct evidence of such a difference may not be readily forthcoming it must be remembered that all the methods of producing methaemoglobin involve a powerful oxidative action, whether the ferricyanide, ferric sulphate or autoxidation method be used. Under such a stress some modification of the protein may well occur.

TABLE 4.

*The perturbation of horse haemoglobin by reagents other than acids or ethanol.*

The perturbations lasted for five minutes.

| Volume of haemoglobin solution. ml. | Conc. of haemoglobin solution. gm./litre. | Temp. C. | Perturbator. | p.c. perturbed. | Restorer. | p.c. restored of that perturbed. |
|---|---|---|---|---|---|---|
| 1 | 72 | 19° | 3 ml. conc. $NH_3$ | 100 | 11 ml. 16 p.c. $H_3PO_4$ | 95 |
| 1 | 72 | 19° | 3 ml. 30 p.c. KCN | 100 | 10 ml. 10 p.c. $NH_4Cl$ | 95 |
| 1 | 150 | 0° | 3 ml. 30 p.c. KCN | 100 | 10 ml. 5 p.c. $H_3PO_4$ | 100 |
| 1 | 150 | 16° | excess solid sodium benzoate | 100 | 10 ml. $H_2O$ | 100 |
| 1 | 150 | 17° | excess solid KI | 20 | 10 ml. $H_2O$ | 100 |
| 1 | 151 | 20° | excess solid sodium benzene sulphonate | 25 | 10 ml. $H_2O$ | 100 |

The difference between methaemoglobin and other derivatives is shown in several ways. At laboratory temperatures the denaturation in saturated urea solution of horse or human haemoglobin or oxyhaemoglobin is very slow. The two cyanmethaemoglobins in neutral solution are unaltered by saturation with urea. No precipitates are formed on dilution and on reduction haemoglobin is, after the first few minutes, the sole product. The cyanmethaemoglobins were perturbed by saturation of their solutions with potassium iodide or sodium benzoate and precipitated on dilution with water. On dissolving the precipitates with dilute ammonia and treating with sodium hyposulphite it was found that scarcely any had been denatured. The methaemoglobins, however, were perturbed by urea, precipitated on dilution of their solutions with water and a test similar to the last showed that 20 p.c. of each protein was denatured. In like manner potassium iodide and sodium benzoate denatured half of each methaemoglobin.

Among other reagents used to perturb or denature haemoglobin were the following: pyridine, phenol, formalin, sodium salicylate, sodium benzoate, potassium iodide, potassium thiocyanate, ammonia and potassium cyanide. The first three have a direct denaturant action and little, if any, restoration can be observed. Sodium salicylate, sodium benzoate, concentrated ammonia, potassium cyanide and potassium iodide changed the haemoglobin into a substance with a spectrum resembling that of haemochromogen, in the case of potassium cyanide that of cyanhaemochromogen. Potassium iodide only perturbed part of the haemoglobin. In all these cases the appropriate restorer (shown in Table 4) completely restored the pigment to haemoglobin. Potassium thiocyanate both oxidized the iron of haemoglobin and denatured it. No restoration could be observed. The wave-lengths of the α bands of the haemochromogens seen on perturbation are given in Table 5. They differ from that of denatured globin haemochromogen.

TABLE 5.

Wave-lengths in mμ of the α bands of haemochromogen-like compounds resulting from the treatment of horse haemoglobin with the reagents indicated.

| Reagent | mμ | Reagent | mμ |
|---|---|---|---|
| Sodium benzoate | 561 | Ammonia | 559 |
| Sodium salicylate | 560 | Potassium iodide | 561 |
| Sodium benzene sulphonate | 560 | Potassium cyanide | 567 |

All the above were restored to haemoglobin. Pyridine produced a haemochromogen with a band at 557. This substance was completely denatured. The spectrum observed with potassium cyanide resembled that of cyanhaemochromogen rather than that of reduced cyanmethaemoglobin, which has a typical haemochromogen spectrum with an intense α band at 565 mμ.

It is known that if a solution of haemoglobin, perturbed by acid, be neutralized suddenly with sodium carbonate or better with sodium phosphate, an insoluble pigment whose spectrum is that of a haemochromogen is formed. The precipitate is denatured by oxygen, but on careful solution in its absence with the minimum of acid or alkali it is restored to haemoglobin. Apart from the denaturation by oxygen a similar precipitate was seen when methaemoglobin was similarly perturbed. In the course of the experiments of this paper a like effect was observed when methaemoglobin but not when haemoglobin or cyanmethaemoglobin, was precipitated with alcohol. It frequently happened that a precipitate which was quite insoluble in water was found when treated with dilute ammonia or ammonia and sodium hyposulphite to be largely composed of restorable protein. When haemoglobin or cyanmethaemoglobin was precipitated with alcohol the native protein could be dissolved away readily in water leaving, in the latter case, some insoluble denatured protein behind. A ready distinction of these complexes of perturbed and denatured blood pigment derivatives from pure denatured derivatives is the ease with which the former break up in dilute ammonia, the native protein going into solution. A precipitate composed solely or mainly of denatured material requires dilute sodium hydroxide to dissolve it. For any derivative the amount of restoration observed decreased with increase of temperature. Though less strongly than with haemoglobin a definite increase in denaturation due to oxygen was observed when methaemoglobin or cyanmethaemoglobin were treated with acid or alcohol. Methaemoglobin is both less soluble than haemoglobin or cyanmethaemoglobin in alcohol-water mixtures and is more readily denatured by alcohol.

TABLE 6.

Wave-lengths in mμ of the bands in the red region of carbon monoxide compounds of green pigment formed from horse haemoglobin in the presence of various promoters.

| Promoter. | Formed with $Na_2S_2O_4$ and $O_2$ | Formed with $Na_2S_2O_4$ and $H_2O_2$ |
|---|---|---|
| Pyridine | 623 | 627 |
| Phenol | 624 | 628 |
| Ethanol | 625 | 629 |
| Hydrazine (no other reducer) | 618 | 624 |
| Sodium benzoate | 621 | 628 |
| Sodium salicylate | 625 | 628 |
| Sodium benzene sulphonate | 624 | 629 |
| Potassium iodide | 620 | 628 |
| Potassium cyanide | 620 | 631 |
| Formaldehyde | 625 | 615 (no $Na_2S_2O_4$) |

The compound formed using hydrazine and oxygen probably contained denatured material.

The second group of experiments consisted in the preparation of green pigments by the repeated desoxygenation of horse oxyhaemoglobin with sodium hyposulphite in the presence of various chemicals (termed "promoters") and its re-oxygenation with atmospheric oxygen. They were performed in test-tubes, the tubes being rotated at a small angle with the horizontal to oxygenate the liquid without undue frothing. In parallel experiments the desoxygenated pigment was at once treated with a few drops of 1 p.c. hydrogen peroxide. In some cases the preliminary desoxygenation was found unnecessary and the mixture of oxyhaemoglobin and promoter was treated directly with hydrogen peroxide. The first chemicals tested as promoters were those in Tables 4 and 5 used in relatively low concentrations. Afterwards a larger range of substances was similarly tested. In nearly every case it was found that two pigments were formed according to whether oxygen or hydrogen peroxide were employed. The absorption

band of the carbon monoxide derivative prepared with oxygen had in general a wave-length a few mμ shorter than that with hydrogen peroxide. The wave-lengths of the bands varied somewhat irregularly for different promoters and even for different specimens prepared using the same promoter. It would appear probable that each of the two green pigments formed through the agency of a given promoter was contaminated to a variable degree by the other. The details are in Table 6. The difference in the two series is probably due to modifications in the vinyl side-chains of the prosthetic group brought about by the prolonged action of sodium hyposulphite and oxygen.

A group of substances that have well-marked disintegrative effects on the blood pigment are aromatic hydroxyl and quinone derivatives. Thus phenol denatures haemoglobin but in lower concentration acts as a promoter of green pigment formation. If α naphthol in a weakly alkaline solution be added to oxyhaemoglobin a portion of the pigment is rapidly converted to alkaline haematin and another part transformed to a green pigment with a band like those obtained through the action of hydrogen peroxide. Naphthoquinone rapidly converts oxyhaemoglobin to a mixture of denatured globin parahaematin and a similar green pigment. In neutral solution quinol turns oxyhaemoglobin to methaemoglobin. In weakly alkaline solution its action is similar to that of α naphthol. In these cases the one substance appears both to act as a promoter and, by interaction with the oxygen of the blood pigment, as a furnisher of the necessary hydrogen peroxide.

TABLE 7.

The separation of the prosthetic group from some green pigments and the wave-lengths of the bands of its carbon monoxide derivative. Horse oxyhaemoglobin, sodium hyposulphite and oxygen were used.

| | Wave-length, mμ of CO compounds of free group. | | | Wave-length, mμ of CO compounds of free group. | |
|---|---|---|---|---|---|
| Phenol | 0·3 g. | | Pyridine | 1 ml. | |
| Oxyhaemoglobin 16 p.c. | 10 ml. | 614 | Oxyhaemoglobin 13 p.c. | 25 ml. | 616 |
| $Na_2CO_3$ 10 p.c. | 4 ml. | | $N.NH_3$ | 10 ml. | |
| $H_2O$ | 20 ml. | | $H_2O$ | 45 ml. | |
| α naphthol | 0·2 g. | | Potassium iodide | 5 g. | |
| Oxyhaemoglobin 13 p.c. | 25 ml. | 612 | Oxyhaemoglobin 2·7 p.c. | 50 ml. | 616 |
| $N.NH_3$ | 20 ml. | | $N.NH_3$ | 20 ml. | |
| $H_2O$ | 40 ml. | | $H_2O$ | 50 ml. | |
| Sodium salicylate | 12 g. | | Hydrazine hydrochloride | 0·5 g. | |
| Oxyhaemoglobin 13 p.c. | 25 ml. | 626 | Oxyhaemoglobin 15 p.c. | 25 ml. | 614 |
| $N.NH_3$ | 10 ml. | | $Na_2CO_3$ 10 p.c. | 4 ml. | |
| $H_2O$ | 45 ml. | | No sodium hyposulphite | | |

The third group of experiments involved the separation from the protein of the prosthetic groups of some of the green derivatives. The methods of their preparation in suitable amount and of the separation of the prosthetic group were essentially those already described for the preparation of cruoratin (Holden, 1945), starting however from oxyhaemoglobin and using a concentration of the promoter found suitable in test-tube experiments. The details and the wave-lengths of the bands of the CO compounds of the prosthetic groups are in Table 7. The yields were always small compared with that obtained from cruoralbin. This appears to be due partly to a further oxidative destruction of the green pigment whose concentration appears to reach a maximum and then to diminish. Other reactions apparently also occur. Bile pigments were always found. In addition to its function as a promoter, cyanide appears to impart a certain small degree of stability to the cruoralbin produced under its influence, impeding its further destruction. Incidentally bile pigments have not been found in solutions of cruoralbin.

The tint of the solution of the green pigments formed in some experiments showed that much of the haem was being broken down in a direction different from that leading either to choleglobin or to cruoralbin. The solutions often developed a slatey-bluish colour—later turning almost black. One of the most striking examples of this was when a small amount of copper sulphate in normal ammonia was used as a promoter. The pigment was extracted from a large preparation with amyl alcohol and acetic acid as usual, the acid removed by washing and the pigments taken into dilute sodium hydroxide. An example may be quoted of another means for the oxidative destruction of haemoglobin, the use of hydrazine. To a 10 p.c. solution of horse oxyhaemoglobin, containing the stroma, was added some hydrazine sulphate dissolved in excess of ammonia. The mixture was placed in a shallow dish at 0° C. for several days. When most of the blood pigment appeared to have been destroyed the pigments were extracted with amyl alcohol and acetic acid. After being removed thence into aqueous ammonia it was chromatographed over barium oxalate. The carbon monoxide compound so obtained had a band of 614 mμ, near to that of cruoraem carbon monoxide, so that its vinyl side-chains had presumably been attacked.

## DISCUSSION.

Any attempt to formulate for these experiments a unifying mechanism or to derive from them some information about the constitution of the blood pigment encounters a serious difficulty at the outset. So much of the constitution of the blood pigment and its immediate derivatives has been rigidly established on the basis of titration curves, determinations of magnetic susceptibility, measurements of reaction velocities at varying temperatures and other physical measurements, that purely chemical facts have to be fitted into this framework as best they can. The prosthetic group, whether ferric or ferrous, is linked to the globin by two iminazolyl groups of histidine molecules placed just too far apart for co-ordination. Any substance, except a simple ion, which combines with the blood pigment or a derivative does so by uniting with the iron atom of the prosthetic group. All other possibilities have apparently been excluded.

Though not all compounds of blood pigment derivatives have covalent linkages around the iron atom the native protein can only be so linked to the prosthetic group when another smaller molecule such as carbon monoxide is also covalently linked to the iron atom of the prosthetic group. The effect of ammonia in diminishing the magnetic susceptibility of haemoglobin and the transitory spectrum seen during the reduction of cyanmethaemoglobin have been pronounced to be proofs of the formation of ammonia haemoglobin and cyanhaemoglobin analogous to carbon monoxide haemoglobin. The spectrum of ammonia haemoglobin was apparently not observed, but it is now seen to be that of a haemochromogen. It is of interest to note that ammonia haemoglobin, unlike carbon monoxide haemoglobin, is denatured by oxygen. That ammonia has a reversible effect on the globin, apart from the formation of ammonia haemoglobin, is shown by the fact that on the addition to it of the first few drops of dilute phosphoric acid, while the solution was still alkaline, most of the protein was precipitated but redissolved on the addition of the rest of the acid which made the solution nearly neutral. The increase in the magnetic susceptibility of alkaline methaemoglobin in the presence of a large amount of ethanol demonstrated the combination of these substances. The alteration in the spectrum of alkaline methaemoglobin (not of haemoglobin as is so often stated in error) in the presence of sodium salicylate proved the denaturation of the former (Anson and Mirsky, 1934). Curiously enough the change of spectrum of methaemoglobin in the presence of sodium fluoride proves the existence of a compound between them and not, as in the preceding instance, of a denaturation though it is equally reversible.

It may well be argued from one or other of the preceding examples that the haemochromogen spectra observed when haemoglobin is in the presence of potassium iodide, sodium salicylate, sodium benzene sulphonate, ethanol, etc., are proof that these substances combine with haemoglobin as does carbon monoxide, or, perhaps, as pyridine does with myoglobin, but not with haemoglobin (Kiese and Kaeske, 1942). It may be here mentioned that if at pH 9 a solution of alkaline horse methaemoglobin be treated with a little dilute formalin solution, also just previously adjusted to pH 9, the spectrum changes at once to that of methaemoglobin, which is then slowly converted to that of alkaline haematin with but little denatured globin parahaematin. On reduction it yields mostly haem with a little denatured globin haemochromogen. By current reasoning this would indicate the formation of a temporary compound of formaldehyde and methaemoglobin with a spectrum resembling that of the latter. Holden and Freeman (1929) showed that formaldehyde greatly diminishes the ability of denatured globin to form parahaematin or haemochromogen. In contrast they

found that formaldehyde has no effect on the formation of parahaematins or haemochromogens by simple iminazole compounds. This has been re-examined and confirmed. Thus each molecule of haem combines with two iminazole groups of native globin while in denatured globin haemochromogen at most with only one. The nature of the second group involved is unknown—apparently it is a primary or secondary amine. If a little phenol be added to a solution of haemin dissolved in dilute sodium hydroxide solution the spectrum changes at once to that of acid haematin though the solution is still alkaline. Whatever be the explanation, one hestitates to add phenols to the already long list of compounds that combine with haematin.

Unlike other compounds under discussion, carbon monoxide haemoglobin has a spectrum resembling that of carbon monoxide haem and of carbon monoxide denatured globin haemochromogen instead of having as might be expected a haemochromogen spectrum. Among the simpler compounds of haem there is but one known which shows a true haemochromogen spectrum without both the iron valencies external to the porphyrin ring being attached to suitable nitrogen atoms. Many years ago Anson and Mirsky (1928), in a paper whose importance seems to have been overlooked, showed that haem combines with hydrogen cyanide in equi-molecular proportions to form a substance showing the visible spectrum typical of a haemochromogen, the sixth co-ordination place around the iron atom being filled with a non-nitrogenous substance or group (water or $OH'$). It has also long been known (see, e.g. Hill, R., 1926, Pauling and Coryell, 1936) that for all other simpler bases such as pyridine or nicotine two molecules of base are required per molecule of haem for the formation of a haemochromogen. The work of Anson and Mirsky permits of the haemochromogen spectra of perturbed haemoglobin being interpreted as due to the co-ordination of the iron atom of the haem with one nitrogen atom of the globin and a non-nitrogenous substance such as are many of the perturbators. It does not much assist in finding a common factor in so assorted a group of substances.

In August, 1946, Mr. J. P. Callaghan communicated to the Australian and New Zealand Association for the Advancement of Science the results of a series of determinations with a Hardy recording spectrophotometer of the absorption spectra of solutions of haem containing cyanide ions. He confirmed the observation of Anson and Mirsky (1928) of the existence of two compounds of haem with cyanide. He showed that the four-banded spectrum seen under certain conditions is due to a mixture of the two compounds. On the assumption that in his experiments all the haem in the "monocyanide" compound is combined with cyanide the maximum $\Sigma$ is 15,100. This assumption has not yet been proved, but, if correct, the $\Sigma$ is only about half that of pyridine protohaemochromogen though the spectra are otherwise similar.

Denaturation has been shown to be a change, involving the rupture of many weak hydrogen bonds, from an almost unique to an exceedingly random type of structure. If the temporary conversion of haemoglobin to compounds with a haemochromogen spectrum is a temporary denaturation rather than a formation with divers reagents of a series of compounds all possessing this structure and spectrum, it is a little difficult to reconcile the extensive changes in the structure of the globin molecule that are implied with the ease with which these peculiar reactions can be reversed. A separate classification is perhaps not altogether unjustified.

While carbon monoxide haemoglobin and nitric oxide haemoglobin are essentially unchanged in structure by molecular oxygen, a much longer list is oxidatively disintegrated by this gas with the formation of various types of break-down products. The list can undoubtedly by greatly extended by anyone

to whom adequate stocks of chemicals are available. It is not easy to see, on current theories, wherein lies the peculiar virtue of carbon monoxide as compared, with say, potassium iodide or cyanide in preventing the disintegration by oxygen of its compound with haemoglobin. The day when the chemist might attempt to describe, if not elucidate, the phenomenon with the help of separate constitutional formulae for these substances differing so in their reactions appears to be over.

It can be seen that sodium hyposulphite, and this holds for most reducing agents, is not so innocent an absorbent of oxygen as it might seem. Since oxyhaemoglobin is dissociable, a reducing agent would be unnecessary for the formation of green pigments if its sole function were to promote the dissociation by reacting with the oxygen dissolved in the solution.

It is attractive to imagine that a perturbing agent forms a dissociable compound with haemoglobin. In view of the probable number of such compounds, of the apparently entirely unrelated natures of the perturbing agents and of the almost uniform haemochromogen spectra of the compounds formed, it becomes increasingly difficult to assign all the responsibility to the iron atom. There, however, it must rest while the existing structure of haemoglobin is maintained.

That the group of reagents under survey can under suitable conditions perturb haemoglobin, assist oxygen to denature oxyhaemoglobin or promote the formation of green pigments would indicate some relation between these reactions. A haemoglobin perturbed is usually too readily denatured by oxygen for it to be, except in a few cases, a very likely intermediate in the formation of a green pigment. Since a lower concentration of a perturbator than is required for a visible degree of perturbation is adequate for the promotion of green pigment formation, one is tempted to assume an intermediate step whereby a little of the haemoglobin is modified in such a way that the molecular oxygen neither unites with the iron atom nor so reacts as to denature the protein, but takes a third course.

## SUMMARY.

A number of substances which effect the denaturation of oxyhaemoglobin convert haemoglobin to substances with a haemochromogen spectrum. In the absence of oxygen the phenomenon can be reversed.

The same substances are among those which promote the formation of green pigments through the oxidative disintegration of haemoglobin.

The list includes ammonia, ethanol, potassium iodide, sodium benzoate, and other aromatic compounds.

The different effects of acid and of ethanol on four derivatives of the blood pigments of horse and of man are described.

## REFERENCES.

Anson, M. L. (1938): Chemistry of Amino-acids and Proteins. Edited by C. L. A. Schmidt. C. C. Thomas, Springfield, Ill., U.S.A.
Anson, M. L. and Mirsky, A. E. (1928): J. gen. Physiol., 12, p. 273.
Anson, M. L. and Mirsky, A. E. (1934): *Ibid.*, 17, p. 399.
Hill, R. (1926): Proc. Roy. Soc. London, 100B, p. 419.
Holden, H. F. (1937): Austral. J. exp. Biol., 15, p. 43.
Holden, H. F. (1945): *Ibid.*, 23, p. 255, 24, p. 107.
Holden, H. F. and Freeman, M. (1929): *Ibid.*, 6, p. 79.
Kiese, M. and Kaeske, H. (1942): Biochem. Z., 312, p. 121.
Lemberg, R. (1943): Austral. J. exp. Biol., 21, p. 239.
Mirsky, A. E. and Pauling, L. (1936): Proc. Natl. Acad. Sci. U.S., 22, p. 439.
Neurath, H. *et. al.* (1943): J. gen. Physiol., 26, p. 513.
Neurath, H., Cooper, G. R. and Erickson, J. O. (1942): J. Phys. Chem., 46, p. 203.
Pauling, L. and Coryell, C. D. (1936): Proc. Natl. Acad. Sci. U.S., 22, p. 159.

# ON THE COLORIMETRIC DETERMINATION OF HAEMOGLOBIN

by HENRY FRANCIS HOLDEN[1]

(From the Walter and Eliza Hall Institute, Melbourne).

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The large number of methods devised, modified, praised and condemned, for the determination of haemoglobin in small samples for clinical or research purposes is perhaps the most eloquent testimony possible to the fact that none has given unqualified satisfaction or is entirely free from obvious disadvantages. This paper records a series of investigations undertaken to see what practicable improvements could be suggested at once to the isolated physician whose determinations are relatively few and to the worker in the busy pathological laboratory who has to endure the daily strain of carrying out a large number of these essential analyses.

These investigations cover three aspects of the problem: (1) the apparatus commonly used, (2) the standardization of the method chiefly employed in this country and (3) a study of a new method which with regard to self-consistency, simplicity and speed seems to deserve serious consideration.

The method used almost exclusively in Australia is the version of the acid haematin method associated with the name of Sahli. Since in this, as in other methods, it often happens that a number of pipettes and dilution tubes are used with one apparatus a survey was made of some of these articles when they were purchased and before they were used. It was found that while the majority of pipettes did not err by more than 2 p.c., individuals were fairly frequently found with enormous errors, amounting in one case to nearly 50 p.c. in excess. Many pipettes were poorly made, having both too large a bore and too large an orifice. With a photo-electric colorimeter sensitive to differences of rather less than one per cent. in the concentration of oxyhaemoglobin present in a solution it was ascertained that with the usual commercial 20 cmm. pipette, even when very carefully handled, there were small but detectable variations in the amount of haemoglobin solution which it was judged to contain. With a specially made bulb type 50 cmm. pipette these variations fell below the discriminatory power of the instrument. These results support various suggestions in the English literature that for exact work 20 cmm. of blood is too small a sample. Some dilution tubes were calibrated and it was found that those specifically designated for a particular brand of haemoglobinometer differed little from one another. There is, however, reason to think that the results in this small series offer too favourable a view of the situation and that had a wider range of makes been available for testing the results would have been less satisfactory. More serious divergences from the correct value have been noticed in the permanent glass standards used.

In analysis it is a sound principle that all secondary methods should be calibrated by the analyst responsible; attention was therefore directed towards the preparation of a derivative of the blood pigment that could be prepared in a central laboratory and whose stability would permit it to be analysed there and conveyed thence to anyone who might desire to purchase some for the standardization of his own haemometer.

Oxyhaemoglobin deteriorates far more rapidly than is generally recognized; the high temperatures and great distances of this country render it useless as a standard. Carbon monoxide haemoglobin was prepared and its stability at 37° C. determined. Where all oxygen had been successfully excluded it proved to be reasonably stable for several days but the destructive effect of small amounts of oxygen in occasional samples and the speed with which it decomposed when once the tube had been opened for the withdrawal of the first batch of samples rendered it unattractive.

The other possible derivative was cyanmethaemoglobin. Since it contains ferric iron it is less liable to oxidative damage than is carbon monoxide haemoglobin, while the cyanide renders it little liable to reduction by the usual run of organic reducing agents. After various trials

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the most satisfactory method of preparation was found to be the following: 100 ml. of fresh human blood were carefully filtered through gauze to remove any fine clots. The cells were then well washed and laked by freezing in carbon dioxide snow. A volume of water equal to that of the laked cells was added and 2·5 ml. each of 10 p.c. ammonium chloride and 10 p.c. potassium cyanide. After careful mixing the fluid was stored in the dark in the laboratory at 16°–20° C. till the bands of oxyhaemoglobin were invisible. It was kept for one night at 37° C. and was then ready for analysis and tests of its stability. At 0° C. such a solution remains almost unchanged for many days and even at 37° C. its deterioration as can be seen from Table 1 is sufficiently slow to permit of its being flown any required distance in perfect confidence.

TABLE 1.

*Determination as acid haematin of cyanmethaemoglobin kept at 37° C. Expressed as a percentage of the original concentration.*

| | Period. | Concentrations found. |
|---|---|---|
| 1. | 3.5.46–13.5.46 | 100, 101, 100, 99 |
| 2. | 20.5.46–29.5.46 | 100, 103, 102, 102 |
| 3. | 21.5.46–29.5.46 | 100, 100, 98, 99 |
| 4. | 7.7.46–23.7.46 | 100, 99, 97, 100, 100 |
| 5. | 15.7.46–25.7.46 | 100, 101, 101, 101, 100 |

The determinations were made with a photo-electric colorimeter on 0·05 ml. samples. A separate tube was opened for each determination.

TABLE 2.

*Determination as acid haematin of carbon monoxide haemoglobin kept at 37° C. Expressed as a percentage of the original concentration.*

| | Period. | Concentrations found. |
|---|---|---|
| 1. | 11.4.46–26.4.46 | 100, 100, 102, 101 |
| 2. | 12.4.46– 3.5.46 | 100, 94, 98, 99, 98, 98 |
| 3. | 9.5.46–29.5.46 | 100, 100, 99, 97, 99, 93, 98 |

Determinations were made as for Table 1.

In the course of this work a comparison was made of the maximum absorption coefficient of twice crystallized horse cyanmethaemoglobin prepared by this method and of that prepared by the action of potassium cyanide on twice crystallized horse methaemoglobin prepared by the autoxidation of oxyhaemoglobin at 37° C. and pH 5·3 with the maximum absorption coefficient of horse cyanmethaemoglobin prepared directly by the action of potassium cyanide and pofassium ferricyanide on oxyhaemoglobin. It was found that the coefficient of the visible band of the first two specimens was some 5–7 p.c. lower than that of the one prepared directly. Besides giving a warning against too great a confidence in the purity of autoxidation methaemoglobin, it shows the necessity for the conduct in the central laboratory of the analysis with the same method that will be applied by the purchaser.

For those who wish to use the acid haematin method in a visual colorimeter a mixture of inorganic salts can be used which imitates very fairly the tint of a solution of acid haematin. Such a procedure has been found moderately satisfactory in a stable artificial light but to be quite unreliable in the changing hues of daylight. By setting the cup containing the acid haematin at a predetermined position and placing the salt mixture in the moveable cup the instrument can be approximately graduated to read directly gm. of haemoglobin per 100 ml. The details will be found in Table 5.

The acid haematin method of haemoglobinometry does not improve on acquaintance. Different derivatives of the blood pigment yield solutions of acid haematin of different depths of colour. It is known to have a high temperature coefficient. The rapid intensification of the colour during the first half hour and the necessity for a prolonged wait for more stable conditions are not attractive. Worse still is the fact, not often realized, that much of the darkening of the solution is due to incipient aggregation of the particles. The cumulative effect of these draw-backs has led to the investigation by several workers of the determination of the blood pigment in the form of alkaline haematin. A comparison standard of inorganic salts has been proposed by Gibson and Harrison (1945). The method suffers from the disadvantage that for both the determination and the preparation of the standard heating under strictly controlled conditions is essential.

During some recent studies on the properties of the blood pigment it was found that cold solutions of certain cleaning materials instantly denature oxyhaemoglobin. A detailed search showed that oleic acid in the form of one of its salts was a very suitable material for this

purpose. Oleic acid was freed from any remaining esters and distilled from a Claisen flask under a pressure not exceeding 0·1 mm. The whole distillation apparatus must be of pyrex glass. The oleic acid and its alkaline solution must be kept in pyrex vessels. Contact with soft soda glass results in the solution becoming turbid after two or three days.

TABLE 3.

*Temperature effects of acid and of cold alkaline haematin methods. The acid haematin was read two hours after mixing. The factors are those by which the observed concentrations must be multiplied to read correctly on an instrument standardized at 16° C.*

| | 0° C. | 30° C. |
|---|---|---|
| Acid | 1·22 | 0·9 |
| Alkaline | 0·99 | 1·0 |

In a pyrex flask two gm. were dissolved in 200 ml. of 0·1 N potassium hydroxide containing 1 ml. of concentrated ammonia. Sodium hydroxide is much less suitable. If properly prepared such a solution remains clear and bright. Twenty cmm. of blood measured into 4 ml. of this potassium oleate yields a solution in which all the pigment is instantly converted to alkaline haematin and the absorption of light remains unchanged for at least half an hour. If a green screen be available the ammonia can advantageously be increased and the potash omitted. In this case a fine parahaematin is formed with a broad band in the green region very suitable for photometry when a certain selection of light is possible. Green glass with a very low transmission of yellow and red rays is widely available on account of its use for signals.

Those who can obtain lauric acid, either by purchase or by fractionation of saponified cocoanut oil, may find it convenient to substitute this acid for oleic. Being a crystalline powder it is more readily handled and dissolves more easily. On account of its lower molecular weight a 0·7 p.c. solution is sufficient. It produces the same depth of colour as does oleic acid. The same precautions as to glassware must be observed as for oleic acid.

TABLE 4.

*Self-consistency of cold alkaline haematin method using a visual colorimeter and artificial light.*

| P.c. of haemaglobin | | P.c. of haemaglobin | |
|---|---|---|---|
| Known. | Found. | Known. | Found. |
| 8·4 | 8·5 | 12·6 | 12·6 |
| 9·45 | 9·5 | 13·75 | 13·65 |
| 10·8 | 10·7 | 14·4 | 14·3 |

A similar method was followed for acid haematin with nearly as consistent results:

TABLE 5.

*Self-consistency of acid haematin method using a visual colorimeter and artificial light.*

| P.c. of haemaglobin | | P.c. of haemaglobin | |
|---|---|---|---|
| Known. | Found. | Known. | Found. |
| 7·85 | 7·95 | 12·9 | 12·95 |
| 10·0 | 10·0 | 13·1 | 13·0 |
| 10·5 | 10·3 | 13·85 | 13·8 |
| 10·6 | 10·4 | 15·3 | 16·0 |
| 11·3 | 11·2 | | |

The salt mixtures were made as follows:

Since, however, the criterion is the human eye, each observer must prepare his own final mixture and calibrate the procedure for himself.

For acid haematin: in 100 ml. of solution 3·75 gm. $CoCl_26H_2O$ 0·0333 gm. $K_2Cr_2O_7$, 5·55 gm. $NiSO_47H_2O$ 0·25 gm. $H_2SO_4$.

For alkaline haematin: in 100 ml. of solution 0·0343 gm. $K_2Cr_2O_7$ 2·08 gm. $KCr(SO_4)_2$ $12H_2O$, 0·643 gm. $CoCl_26H_2O$ 0·25 gm. $H_2SO_4$.

The solution for use with alkaline haematin must not be heated during its preparation.

For a proper standardization of the alkaline haematin method the only possible blood pigment derivative is carbon monoxide haemoglobin prepared under the most stringent conditions to ensure freedom from oxygen. No other course seems practicable since potassium oleate does not transform cyanmethaemoglobin into alkaline haematin. It must also be remembered that the cold alkaline haematin method produces from an equal amount of oxyhaemoglobin an appreciably deeper colour than does the method employing heat.

Carbon monoxide haemoglobin has a rather higher "colour value" in this cold alkaline haematin method than has oxyhaemoglobin but the difference is less than in the acid haematin methods. The temperature coefficient is so small over the usual range of laboratory temperatures that it may usually be neglected.

The self-consistency of the cold alkaline haematin method was tested as follows: Various solutions containing known amounts of oxyhaemoglobin denatured in potassium oleate were placed successively in the "fixed" cup of a colorimeter illuminated with artificial light. Using the first solution made from 15·1 gm. p.c. haemoglobin the position was determined of the fixed cup which correctly matched the "movable" cup containing the inorganic salts when the latter was set to 15·1 mm. The other known solutions were successively placed in the fixed cup set to the same position and the matching position of the movable cup found for each. As can be seen from Table 4, the readings of these positions correspond nearly to the known concentrations of oxyhaemoglobin used.

## DISCUSSION.

The experiences described in this paper strongly support the view already stated in many quarters that calibration of the glass parts of haemoglobinometers is a service which should be available through the standards laboratory of a nation. The supply through the chief health authority of standard blood pigment derivatives, that analysts who so desire may verify the quality of their work, would seem to be a reasonable extension of this principle. It has been shown that such derivatives can be prepared and are sufficiently stable for practical purposes.

The cold alkaline haematin method of haemoglobin determination has been shown to be sufficiently inexpensive and self-consistent for its advantages in speed and simplicity over the acid haematin method to deserve serious consideration by those who are not in a position to employ elaborate apparatus for haemoglobinometry.

In large institutions there might be an advantage in assigning the determinations of haemoglobin to the biochemist wherever possible, particularly if any of the methods using more elaborate apparatus are contemplated. After nearly a year's experience with a sensitive photo-electric colorimeter of the Evelyn direct-reading type the following conclusions may be stated: A photo-electric colorimeter will only yield results that justify its cost in the hands of a worker well accustomed to the meticulous care of a visual colorimeter that is necessary to obtain exact results from it. The exact reading of instrument dials and the carrying out of the rigid drill necessary cause an appreciable fatigue and it was found inadvisable to perform more than ten determinations without a short rest and change of activity. The short life of essential portions, e.g. non-standard electric bulbs of imported instruments renders a very good stock of spare parts most desirable.

## SUMMARY.

An examination of a number of 20 cmm. blood pipettes stresses the need for their individual calibration before use.

The preparation of cyanmethaemoglobin for the standardization of the acid haematin method of haemoglobinometry is described.

A cold alkaline haematin method is outlined. Its advantages of speed, simplicity, self-consistency render it superior to the acid haematin method.

Matching inorganic salt solutions for the acid haematin and cold alkaline haematin methods are described and the standardization of the latter discussed.

*Acknowledgment.* For the competent and faithful performance of many analyses I am indebted to Mr. R. H. Leslie of this Institute.

## REFERENCE.

Gibson, Q. H. and Harrison, D. C. (1945): Biochem. J., 39, p. 490.

# THE EFFECT OF ADRENALECTOMY ON THE RESPONSES OF THE GUINEA-PIG TO INJECTIONS OF THYROTROPHIC HORMONE

## (WITH ESTIMATIONS OF LIVER GLYCOGEN AND ASCORBIC ACID[1])

by A. R. ROBINSON AND V. M. TRIKOJUS[2]

(From the Department of Medicine, University of Sydney).

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In a previous report by one of us (Robinson, 1941), the evidence indicating the rôle of the adrenal glands in the response of animals to injections of thyrotrophic hormone was briefly considered, and a technique for completely adrenalectomizing guinea-pigs was described, together with the routine which was found most suitable for the maintenance of the animals post-operatively.

In the present communication are detailed experiments which were conducted to study certain specific responses of the guinea-pig to injections of thyrotrophic hormone after the complete removal of adrenal tissue. These experiments were made with reference to those changes after injections of thyrotrophic hormone, which previous work had indicated were useful indices of the animal's sensitivity to the injections—namely, the consecutive thyroid histological changes, and the variations in the glycogen and ascorbic acid content of the liver (see also Crabtree and Trikojus, 1946). On account of its more sensitive thyroid gland, the immature guinea-pig is more suited than the rat for studies of the thyrotrophic hormone.

More detailed investigation of the thyroid histological changes in various species, produced by both anterior pituitary preparations and purified thyrotrophic hormone, has shown in the hands of several workers (e.g. Siebert and Smith, 1930; Loeb and Friedman, 1931; Junkmann and Schoeller, 1932; Hertz and Kranes, 1934; Collip and Anderson, 1934; Loeser, 1936a) that, except in the case of the duck (Schockaert, 1932 )and sparrow (Miller, 1938), the appearance of excessive stimulation of the thyroid gland cannot be maintained, but is gradually replaced by the picture of a less active gland, which finally may present a phase of hyper-involution. This subsidence of the morphological changes in the gland associated with functional hyperactivity is accompanied by a regression of all the changes in the animal secondarily dependent on it—in other words, the animal becomes refractory to the administration of the hormone.

The development of resistance has not been satisfactorily explained. Substances inhibitory to thyrotrophic hormone were first demonstrated in, and isolated from, the blood of rats made refractory to thyrotrophic hormone by prolonged injections (over several weeks) by Collip and Anderson (1934); similar inhibitory or neutralizing substances have since been reported by several workers to occur even in normal blood (for literature see Thompson, 1941). Such neutralizing activity protecting the animal against thyrotrophic hormone was ascribed by Collip to an antithyrotrophic hormone; but it is not certain that the protection is due to a single substance, nor is the chemical nature of the protective agent at all clear. Werner (1938) stated that the refractory state is simply an immune response to proteins associated with the extracts, basing his claims on experiments in which he prepared a flavianate extract of thyro-

[1]This investigation was generously aided by a grant from the National Health and Medical Research Council to Professor C. G. Lambie and was performed during the tenure of one of us (A.R.R.) of the Marion Clare Reddal Scholarship in Medicine. The experimental work was carried out in the period 1940–1941.

[2]Present address, Department of Biochemistry, University of Melbourne.

trophic hormone from beef anterior pituitary, which was found capable of maintaining a persistent stimulating effect in guinea-pigs for periods up to 88 days, as shown by basal metabolic rate determinations, evaluation of total thyroid iodine content and thyroid histological changes.

Other factors which have been suggested as influencing the development of resistance to thyrotrophic hormone include an endogenous regulation of hormone balance (Loeser, 1934), an alteration in the reactivity of the thyroid gland (Loeser, Ruland and Trikojus, 1938), and even a reversal of the action of thyrotrophic hormone as a result of the stimulation of thyroxine secretion (Mahaux, 1938).

Whatever the real explanation for the development of resistance, it may be that the adrenals are linked with it in some way, since adrenal hypertrophy following injections of thyrotrophic hormone first develops after a latent period of 4-5 days (Loeser, 1933), but persists beyond the development of a refractory state by the animal, and appears to become aggravated with the continuance of the injections (Loeser, 1934; Trikojus and Ellis, 1939); contrast however, Schmeckebier (1934). It has been pointed out already (Robinson, 1941) that adrenal hypertrophy has been found by most investigators to depend on the concurrent presence of thyroid tissue. The reason for adrenal hypertrophy following injections of thyrotrophic hormone has usually been attributed to the stimulating effect of increased secretion of thyroxine (Loeser, 1933; McQueen-Williams, 1934; Trikojus and Ellis, 1939), or to the concurrent presence of a separate corticotrophic factor in thyrotrophic preparations (Collip, 1935, 1938). Grollman (1936) drew attention to the sensitivity of the adrenals to the injection of foreign protein material, such as might be present in even highly purified pituitary preparations; and a hypertrophic adrenal response under such circumstances might be presumed to be contingent on the presence of thyroid tissue, without its possessing any causal relationship to progressive thyroid histological changes. Werner (1938) was of the opinion that his successful maintenance of thyroid activation and renewal of thyroid stimulation in an animal previously rendered refractory effectively ruled out an interaction between various endocrine glands as an explanation of the development of a refractory state; adrenal changes were not, however, recorded in his experiments.

Although the present consensus of opinion is that the development of a refractory state towards injections of pituitary hormones results from the formation of immune bodies, of a special type, towards the foreign protein in the extracts used (Thompson, 1941; see also McQuillan and Trikojus, 1946), the evidence is not unequivocal, and it seemed desirable to observe whether the complete removal of adrenal tissue had any influence upon thyroid involution following prolonged treatment with heterologous thyrotrophic hormone. Baumann and co-workers (e.g. Baumann and Marine, 1945) have emphasized the existence of an intimate functional relationship between the thyroid and adrenal glands.

It is known that the liver glycogen stores are depleted by injections of thyrotrophic hormone. Thus Eitel and Loeser (1932) found that the liver glycogen commenced to fall after a latent period of four days, reached a stage of almost complete depletion after ten days, and then gradually returned to normal. Loeser 1936, b) drew attention to the fact that there was a close parallelism in time relationship between the development of the maximal histological changes in the thyroid gland and the maximal fall in liver glycogen. He was able to demonstrate, moreover, that serum rich in antithyrotrophic substance prevented the fall in liver glycogen resulting from thyrotrophic hormone, but not that resulting from thyroxine. Any difference in the response of the adrenalectomized as compared with the normal animal to the injection of thyrotrophic hormone might be expected therefore to be reflected in the liver glycogen values. Evaluations of the latter in adrenalectomized animals subjected to thyrotrophic hormone injections have consequently been made, as also have determinations of the liver ascorbic acid.

## MATERIALS.

The animals consisted of immature guinea-pigs of both sexes, derived from one of two stock sources, uniformly maintained under the conditions already detailed (Robinson, 1941). Both normal and adrenalectomized animals were subjected to the same routine care and maintenance.

The thyrotrophic hormone contained 10 Junkmann-Schoeller units per mg. (Junkmann and Schoeller, 1932) and was free from gonadotrophic, growth and lactogenic principles. It was not tested for the presence of a separate adrenocorticotrophic factor. For administration, the hormone was dissolved in 0·9 p.c. saline and given subcutaneously at the back of the animal's neck, to ensure slower absorption than that resulting from intraperitoneal administration. The desoxycorticosterone acetate (DOCA) dissolved in arachis oil or as "Cortenil" was administered subcutaneously according to the method already described (Robinson, 1941).

All injections were given early in the morning, the DOCA being given first. Immediately after the injections, the animals received orally by pipette 1 ml. of a solution containing 10 p.c. saline with 0·8 p.c. sodium citrate.

## EXPERIMENTAL PROCEDURE.

Early attempts to study groups of adrenalectomized guinea-pigs receiving thyrotrophic hormone in addition to a maintenance dose of DOCA were unavailing (see Discussion). The animals were successfully operated upon, and appeared to be surviving in a normal manner on a maintenance dose of DOCA, but following the commencement of thyrotrophic hormone administration, a high proportion of the animals succumbed, usually within three to seven days. This applied whether thyrotrophic hormone injections were commenced either a short (e.g. four days) or a relatively long (e.g. twelve days) time after the complete adrenalectomy; and whether the daily thyrotrophic hormone injections were small (e.g. 2·5 units/day) or large (e.g. 20 units/day). Eventually, however, animal groups of sufficient size to allow the study in completely adrenalectomized animals of both the changes in the thyroid glands following prolonged injection of the thyrotrophic hormone, and the variation in the liver glycogen and ascorbic acid content, were obtained.

### *Prolonged Daily Injection of 2·5 Units Thyrotrophic Hormone.*

A group of twelve guinea-pigs was completely adrenalectomized and maintained with 0·2 mg. DOCA and adjuvant measures already detailed, for periods varying from six to nine days[3] before commencing the daily administration to six of the group of 2·5 units thyrotrophic hormone. Owing to the restricted supply of DOCA available, the period of thyrotrophic hormone injections had to be limited to twenty-two days: but the work of Trikojus and Ellis (1939), using the same batch of hormone, has indicated that, with the small thyrotrophic hormone dosage employed, this period would be sufficiently long to permit the appearance of regressive thyroid histological changes following the initial phase of stimulation in normal guinea-pigs. Six normal animals of approximately the same weight, injected with the same dosage of thyrotrophic hormone over the same period, acted as controls to the adrenalectomized animals; and three guinea-pigs injected with the same dosage for a short period (six days) established the potency of the thyrotrophic hormone preparation used. All animals were despatched by a blow on the neck.

### *Liver Glycogen and Ascorbic Acid Content in Adrenalectomized Animals Following Daily Injection of 20 Units Thyrotrophic Hormone.*

A group of twenty-seven guinea-pigs was completely adrenalectomized, the second operation being performed on each of nine unilaterally adrenalectomized guinea-pigs on three successive days. Eighteen of the animals received 0·2 mg. DOCA commencing the morning after complete adrenalectomy, and were maintained by adjuvant general and dietary measures; the remaining nine animals received only four daily doses of DOCA (0·2 mg., commencing the morning after complete adrenalectomy) and were subsequently treated only by general and dietetic measures (including saline and citrate), but received no additional DOCA. Commencing on the morning of the fourth day after complete adrenalectomy, nine of the eighteen animals still receiving DOCA were daily injected, in addition, with 20 units thyrotrophic hormone. A group of six normal animals under the same general conditions (not, however, receiving DOCA, saline or thyrotrophic hormone) was kept as controls.

The adrenalectomized animals were given either fourteen or fifteen daily injections of thyrotrophic hormone, this being comparable to the period found by Eitel and Loeser (1932) to correspond to the greatest depletion of liver glycogen following thyrotrophic hormone injections, and these animals were then sacrificed in two groups along with the controls on two successive days. Last injections were administered on the morning of sacrifice; all animals were fed right up to the time of despatch, as previous experiments had proved the impracticability of fasting the adrenalectomized animals. These earlier attempts to fast the adrenalectomized guinea-pigs resulted in their death after three or four hours in hypoglycaemic spasms. On the day preceding sacrifice, each guinea-pig was given orally 100 mg. ascorbic acid in distilled water

[3]Two members of the group had been adrenalectomized approximately one month earlier, and maintained on DOCA in the intervening period.

TABLE 1.

*Prolonged administration (22 Days) of thyrotrophic hormone to adrenalectomized guinea-pigs maintained on desoxycorticosterone acetate.*

| Group No. and Treatment. | G.-pig No. and Sex. | Initial weight* (gm.) | Final weight (gm.) | Thyr. H. injected (total units). | Thyroids (mg./100gm. body weight) | Fate of animal and PM comment. | Thyroid histology. | Adrenal remnants. |
|---|---|---|---|---|---|---|---|---|
| I. | | | | | | | | |
| Adrenalectomized animals + DOCA (0·2 mg.) + Thyr. hormone (2·5 units) daily | 23 (F) | 242 | 301 | 55 | 27·7 | Sacrificed; early consolidation of lungs. | Sl. activity; colloid reaccumulation incomplete. | Neg. |
| | 31 (M) | 360 | 413 | 55 | 13·1 | Sacrificed | Resting gland | Neg. |
| | 61 (F) | 264 | 341 | 55 | 13·1 | Sacrificed | Resting gland | Neg. |
| | 63 (F) | 260 | 348 | 55 | 12·3 | Sacrificed | Resting gland | Neg. |
| | 70 (F) | 264 | 455 | 55 | 12·3 | Sacrificed | Resting gland | Neg. |
| | 209 (M) | 247 | 372 | 55 | 14·2 | Sacrificed | Resting gland blood in follicles | Neg. |
| II. | | | | | | | | |
| Adrenalectomized animals + DOCA (0·2 mg.) daily | 26 (F) | 228 | 276 | — | 12·7 | Sacrificed | Resting gland | Neg. |
| | 54 (M) | 231 | 233 | — | 17·2 | Died 6th day; congestion of lungs. | — | Neg. |
| | 56 (M) | 292 | 283 | — | 13·1 | Died 5th day; congestion of lungs. | — | Neg. |
| | 75 (F) | 248 | 239 | — | 10·5 | Died 7th day; congestion of lungs. | — | Neg. |
| | 41 (F) | 398 | 395 | — | 11·6 | Sacrificed | Resting gland | Neg. |
| | 271 (M) | 246 | 233 | — | — | Died 2nd day | — | Neg. |
| III. | | | | | | | | |
| Normal animals + Thyr. hormone (2·5 units) daily | 18 (M) | 209 | 354 | 55 | 11·9 | Sacrificed | Resting gland | |
| | 47 (M) | 215 | 340 | 55 | 15·0 | Sacrificed | Resting gland | |
| | 50 (F) | 208 | 303 | 55 | 9·9 | Sacrificed | Resting gland | |
| | 55 (F) | 229 | 302 | 55 | 13·9 | Sacrificed | Resting gland | |
| | 59 (M) | 217 | 347 | 55 | 11·0 | Sacrificed | Resting gland; blood in follicles | |
| | 227 (M) | 205 | 337 | 55 | 18·1 | Sacrificed | Sl. activity; colloid reaccumulation incomplete; marked congestion | |
| IV. | | | | | | | | |
| Normal animals + Thyr. horm. (2·5 units) daily *for 6 days* | 65 (F) | 242 | 223 | 15 | 23·8 | Sacrificed | Active; colloid pale and vacuolated; epithelium cubical; early papillary infolding | |
| | 69 (F) | 240 | 240 | 15 | 18·3 | Sacrificed | | |
| | 72 (F) | 231 | 227 | 15 | 18·0 | Sacrificed | | |

* At pre-operative stage.

Note: No adrenalectomized guinea-pigs were available at the time of this experiment for the purpose of observing directly the effect on the thyroid histology of the administration of 2·5 units of thyrotrophic hormone for six days. However, in an earlier experiment, similarly planned, each of four completely adrenalectomized animals, which had died within five or seven days of the receipt of 2·5 units of the hormone daily and from which the thyroid glands had been removed soon after death, exhibited thyroidal activity comparable with those of Group IV above.

to ensure the availability to the tissues of a plentiful supply of this substance at the time of despatch (cf. Crabtree and Trikojus, 1946).

The animals were killed by a blow on the back of the neck. The liver of each animal was removed at once, freed of adherent tissue and excess moisture and portions were immediately weighed and then appropriately disposed of for the estimation of glycogen and ascorbic acid content respectively. Glycogen was subsequently estimated by the modification of Pflügers method described by Eitel and Loeser (1932) the glucose in the hydrolysate being determined by the Bertrand method. Ascorbic acid was estimated by the adaptation of the technique of Birch, Harris and Ray (1933) described by Loeser and Trikojus (1937).

Complete post mortem examinations were performed on each animal in both experiments, adrenal sites being particularly carefully scrutinized and suspicious tissue submitted for histological examination. Thyroids were weighed in every case and submitted for histological examination (eosin-haemotoxylin stain).

TABLE 2.

*Liver glycogen and ascorbic acid following administration of thyrotrophic hormone to adrenalectomized guinea-pigs maintained on desoxycorticosterone acetate.*

| Group No. and treatment. | G.-pig No. and Sex. | Initial weight (gm.) | Final weight (gm.) | Thyr. H. injected (total units). | Liver: Thyroids (mg./100gm. body weight). | Liver: Glycogen (gm./100gm. liver). | Liver: Ascorbic acid (mg./gm. liver). | Fate of animal and P.M. comment. | Thyroid histology. | Adrenal remnants. |
|---|---|---|---|---|---|---|---|---|---|---|
| I.* | | | | | | | | | | |
| Adrenalectomized animals+DOCA (0·2 mg.) daily for *4 days only* after 2nd operation | 22 (M) | 321 | 390 | — | 9·5 | 0·79 | 0·33 | Sacrificed | Resting gland; (marked congestion). | Neg. |
| | 71 (F) | 261 | 232 | — | 9·1 | — | — | Died 8th day | — | Small remnant (left) |
| | 136 (F) | 339 | 293 | — | 14·6 | — | — | Died 8th day | — | Neg. |
| | 34 (M) | 260 | 244 | — | 11·1 | — | — | Died 7th day | — | Neg. |
| | 76 (F) | 217 | 219 | — | 10·9 | — | — | Died 5th day | — | Neg. |
| | 452 (M) | 284 | 266 | — | 10·5 | — | — | Died 4th day; blood in stomach | — | Neg. |
| | 25 (M) | 232 | 340 | — | 10·3 | 1·39 | 0·23 | Sacrificed | Resting gland | Neg. |
| | 130 (M) | 299 | 282 | — | 10·6 | — | — | Died 7th day | — | Neg. |
| | 215 (F) | 214 | 223 | — | 12·1 | — | — | Died 5th day | — | Neg. |
| II. | | | | | | | | | | |
| Adrenalectomized animals+DOCA (0·2 mg.) daily throughout experiment | 9 (M) | 334 | 338 | — | 11·8 | — | — | Died 11th day; congestion of lungs | — | Neg. |
| | 17 (F) | 331 | 353 | — | 12·2 | 0·83 | 0·29 | Sacrificed | Resting gland | Neg. |
| | 72 (M) | 255 | 327 | — | 8·8 | 1·18 | 0·31 | Sacrificed | Resting gland | Neg. |
| | 42 (F) | 220 | 260 | — | 11·2 | — | — | Died 11th day; congestion of lungs | — | Neg. |
| | 57 (F) | 318 | 366 | — | 13·9 | 0·00 | 0·28 | Sacrificed | Resting gland; marked congestion. | Neg. |
| | 205 (F) | 266 | 305 | — | 12·5 | 0·22 | 0·24 | Sacrificed | Resting gland; marked congestion. | Neg. |
| | 3 (M) | 269 | 328 | — | 9·2 | 0·37 | 0·31 | Sacrificed | Resting gland; marked congestion. | Neg. |
| | 14 (M) | 217 | 285 | — | 4·2 | 0·37 | 0·21 | Sacrificed | Resting gland | Neg. |
| | 257 (M) | 249 | 273 | — | 11·7 | 0·35 | 0·20 | Sacrificed | Resting gland; sl. congestion. | Neg. |
| Mean (surviving animals): | | | | | 11·4 | 0·47 | 0·26 | | | |

TABLE 2 (continued).

| Group No. and treatment. | G.-pig No. and Sex. | Initial weight (gm.) | Final weight (gm.) | Thyr. H. injected (total units). | Liver: Thyroids (mg./100g.m. body weight). | Liver: Glycogen (gm./100gm. liver). | Liver: Ascorbic acid (mg./gm. liver). | Fate of animal and P.M. comment. | Thyroid histology. | Adrenal remnants. |
|---|---|---|---|---|---|---|---|---|---|---|
| III. | | | | | | | | | | |
| Adrenalectomized animals+DOCA (0·2 mg.) +Thyr. hormone (20 units) daily | 8(F) | 300 | 280 | 60 | 14·2 | — | — | Died 3rd day; congestion of lungs | — | Neg. |
| | 251 (M) | 323 | 325 | 300 | 38·1 | 0·15 | 0·39 | Sacrificed | Very active gland; colloid pale and scanty; columnar epithelium; papillary infolding. | Neg. |
| | 298 (M) | 368 | 376 | 300 | 35·4 | 0·11 | 0·44 | Sacrificed | Active gland; less active than 251. | Neg. |
| | 12 (F) | 247 | 287 | 280 | 46·3 | 0·05 | 0·48 | Sacrificed | Very active, as 251. | Neg. |
| | 28 (F) | 314 | 293 | 60 | 14·1 | — | — | Died 3rd day | — | Neg. |
| | 39 F) | 248 | 267 | 280 | 47·6 | 0·00 | 0·37 | Sacrificed | Active; as 298 | Neg. |
| | 33 (M) | 251 | 238 | 180 | 30·3 | — | — | Died 9th day | — | |
| | 84 (F) | 230 | 243 | 280 | 37·0 | 0·00 | 0·38 | Sacrificed | Very active; as 251. | Neg. |
| | 235 (M) | 257 | 282 | 280 | 33·3 | 0·08 | 0·37 | Sacrificed | Active; as 298. | Neg. |
| Mean (surviving animals): | | | | | 40·5 | 0·07 | 0·40 | | | |
| IV. | | | | | | | | | | |
| Normal control animals | 74 (F) | — | 315 | — | 12·4 | 1·19 | 0·28 | — | Resting gland | — |
| | 53 (M) | — | 319 | — | 11·9 | 1·50 | 0·32 | — | Resting gland | — |
| | 296 (F) | — | 280 | — | 6·8 | 1·30 | 0·27 | — | Resting gland | — |
| | 16 (F) | — | 320 | — | 9·7 | 1·02 | 0·32 | — | Resting gland | — |
| | 20 (M) | — | 318 | — | 12·6 | 2·12 | 0·29 | — | Resting gland | — |
| | 286 (F) | — | 336 | — | 8·0 | 1·86 | 0·33 | — | Resting gland | — |
| | Mean: | | | | 10·2 | 1·50 | 0·30 | | | |

* Mean values not given for Group I as only two animals survived. This group included to check the efficacy of DOCA in the adrenalectomized animal.

## RESULTS AND DISCUSSION.

The treatments and the results obtained from both experiments have been summarized in Tables 1 and 2. The data from the experiment, the results of which are set out in Table 2, were analysed statistically (Table 3). To assess the effects of treatment on all surviving animals, analysis of variance methods were applied, and the ratio of the difference between any two means to its standard error calculated and referred to the standard $t$-table for 16 degrees of freedom. Where the values of this ratio corresponded to $P \leq 0{\cdot}05$, the two means concerned were regarded as being significantly different.

Table 3 shows the mean results for each group, the standard error (S.E.) of this mean calculated from the "within groups" variance in the analysis of variance, and the significant differences between means. These significant differences point to the following conclusions.

(1) Adrenalectomy alone does not alter the thyroid weight, but this is increased by the administration of thyrotrophic hormone.

(2) The values for liver glycogen are higher in the control animals than in either of the two groups of adrenalectomized animals (Groups II and III): the administration of thyrotrophic hormone further depresses the liver glycogen in the adrenalectomized animal.

(3) Adrenalectomized animals receiving thyrotrophic hormone show a significantly higher capacity to concentrate ascorbic acid in the liver than do animals not receiving hormone whether adrenalectomized or not.

TABLE 3.

*Administration of thyrotrophic hormone (20 units/day) to adrenalectomized guinea-pigs and the effect on thyroid weights, liver glycogen and liver ascorbic acid. Statistical treatment of data in Table 2. Group II. Adrenalectomized controls (+ DOCA). Group III. Adrenalectomized plus thyrotrophic hormone. Group IV. Normal controls.*

| Group No. | No. in sample. | Thyroid weights (mg./100 gm. wt.) Mean. | S.E. | Liver glycogen (gm./100 gm. liver). Mean | S.E. | Liver ascorbic acid (gm./100 gm. liver). Mean | S.E. |
|---|---|---|---|---|---|---|---|
| II | 7 | 11·36 | 1·42 | 0·474 | 0·129 | 0·263 | 0·015 |
| III | 6 | 39·62 | 1·53 | 0·065 | 0·139 | 0·405 | 0·016 |
| IV | 6 | 10·23 | 1·53 | 1·498 | 0·139 | 0·302 | 0·016 |

Significant differences between means.

| | Diff. | S.E. | t | P | Diff. | S.E. | t | P | Diff. | S.E. | t | P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| IV–II | — | | | | + 1·024 | 0·189 | 5·412 | <·001 | — | | | |
| IV–III | — 29·39 | 2·17 | 13·57 | <·001 | + 1·433 | 0·196 | 7·302 | <·001 | — 0·103 | 0·023 | 4·441 | <·001 |
| II–III | — 28·26 | 2·09 | 13·54 | <–001 | + 0·409 | 0·189 | 2·163 | <·05 | — 0·142 | 0·022 | 6·339 | <·001 |

*Development of Resistance.*

Following the prolonged injection of small doses of thyrotrophic hormone, no difference in the rate of development of thyroid involution could be demonstrated between normal and adrenalectomized guinea-pigs. Under the conditions of the experiment, the mechanism of the development of resistance is as efficient in the adrenalectomized as in the normal animal, and therefore cannot be related to adrenal function. Figs. 1–4 illustrate the histological appearance of representative thyroid glands of animals from the groups recorded in Table 1.

*Liver Glycogen.*

The depletion of liver glycogen in adrenalectomized animals maintained on DOCA is severe, but administration of thyrotrophic hormone produces further depletion amounting almost to complete exhaustion of liver glycogen stores. The impossibility of fasting these animals for any period of time at all is apparent. This fact was made clear in earlier attempts to determine liver glycogen and ascorbic acid values in adrenalectomized animals after short periods of fasting (12 hours). The animals died in hypoglycaemic convulsions within a few hours of the withdrawal of food, before any experimental determinations could be made.

Adrenalectomy is followed by a profound disturbance of carbohydrate metabolism, liver glycogen values falling practically to zero in these animals after short periods of fasting (e.g. Silvette and Britton, 1936). It would seem from the above results that adrenalectomized animals maintained in an apparent state of health by DOCA are still capable of only very limited glycogenesis. Recent work on desoxycorticosterone and the various active crystalline and non-crystalline fractions, which may be isolated from the adrenal cortex, help to explain why DOCA does not correct the error in carbohydrate metabolism following adrenalectomy. Thus twenty crystalline substances each possessing the same 4-ring system have been isolated from the adrenal cortex; and seven of these have physiological activity (Mason, 1940). The major portion of the total physiological activity of crude extracts remains in the mother-liquor and is obtainable only as an amorphous concentrate (Cortin) representing 90 p.c. of the total activity (Wells and Kendall, 1940a). In a study of the effect of various adrenal cortical fractions and synthetic desoxycorticosterone on phlorhizinized-adrenalectomized rats, Wells and Kendall

(1940b) found that corticosterone and compound E would maintain the quantity of glucose and nitrogen excreted at the same level as before adrenalectomy; but that DOCA, whilst increasing the amount of glucose and nitrogen excreted, did not prevent the animals dying in convulsions in forty-eight hours. In another study Wells (1940) showed that corticosterone and compound E would restore the rate of gluconeogenesis in adrenalectomized-phlorhizinized rats; DOCA and the amorphous fraction failed to do so even in large doses. Similarly, Long, Katzin and Fry (1940) found that DOCA had only a feeble action in restoring the level of glycosuria after adrenalectomy in partially depancreatized rats. Grattan and Jensen (1940) found DOCA ineffective in combating the hypoglycaemia and liver glycogen depletion following insulin injections. It appears that DOCA does not provide complete hormonal substitution following adrenalectomy, because of its inability to correct the defect in carbohydrate metabolism (cf. also Olson, Thayer and Kopp, 1944). This finding, however, does not affect the interpretation of the above experiments that the thyrotrophic hormone exerts its customary effect (see e.g. Crabtree and Trikojus, 1946) on the liver glycogen in adrenalectomized animals.

*Liver Ascorbic Acid.*

The higher values of liver ascorbic acid associated with the administration of thyrotrophic hormone to adrenalectomized guinea-pigs is in line with the observations of Crabtree and Trikojus (1946), on normal animals receiving the same doses of the pituitary hormone for approximately the same period (see also Loeser and Trikojus, 1937; Trikojus and Ellis, 1939). Watanabe (1939) has suggested that the presence of ascorbic acid is necessary for glycogen synthesis in the liver. However, on this supposition, the reason for the elevated values of liver ascorbic acid in the adrenalectomized hormone-treated group in our experiments is not clear, particularly as there was no significant difference between the values for the adrenalectomized and normal control groups, whereas the glycogen values were in marked contrast.

*Thyroids*:

Histological examination of the thyroid glands revealed marked physiological activity in the case of the adrenalectomized animals receiving large doses of thyrotrophic hormone, corresponding in degree to the appearance of the histological picture in normal animals subjected to equivalent thyrotrophic hormone dosage. The thyroids of the adrenalectomized animals not in receipt of the pituitary hormone frequently exhibited an unusual degree of congestion, but showed no signs of activity (see Figs. 2, 5, and 6; also comments in Tables 1 and 2).

## *General Conclusions.*

In the particulars studied, the adrenalectomized animal has behaved in a manner comparable to the normal in its response to thyrotrophic hormone injections. The resistance to prolonged injections of small doses of the thyrotrophic hormone is unaltered after adrenalectomy; the liver glycogen in adrenalectomized animals injected with larger doses of thyrotrophic hormone is almost completely exhausted after 14–15 days, the histological thyroid picture at this time is one of marked activity and the liver ascorbic acid values are raised in adrenalectomized animals receiving thyrotrophic hormone as compared with those that are not.

In view of these findings, the suggestion that the adrenals play a rôle in the development of resistance to thyrotrophic hormone administration must be rejected; and the results show that the adrenals cannot be the site of an indispensable intermediary process between the absorption of vitamin C and its availability to the tissues, confirming the results of Perla and Sandberg (1937). Adrenal hypertrophy following thyrotrophic hormone injections cannot be attributed either to the development of a protective function against thyrotrophic hormone injections, or to the incidental changes in the metabolism of ascorbic acid. The adrenalectomized animal responds to thyrotrophic administration qualitatively in the same manner as the normal.

The reasons for our early experimental failures in studying adrenalectomized animals maintained on DOCA when subjected to thyrotrophic hormone injections cannot be stated with certainty. It may be pointed out, however, that these earlier attempts were conducted during the late winter and early spring months when

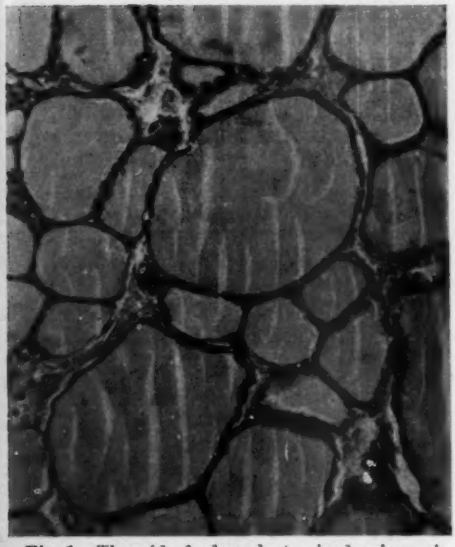


**Fig. 1.** Thyroid of adrenalectomized guinea-pig (26F) maintained on DOCA (control to 31M) (Fig. 2). The appearance is that of a resting gland. Magnification × 180.

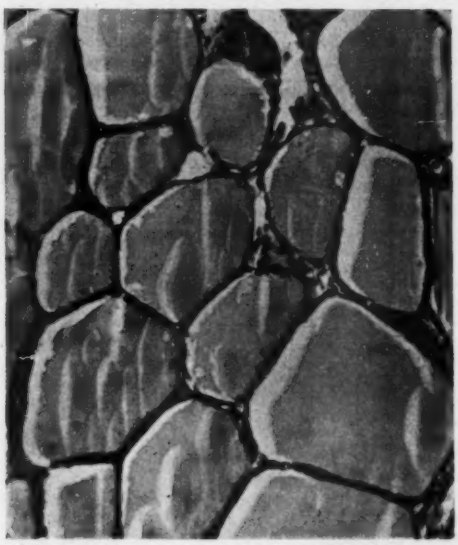


**Fig. 2.** Thyroid of adrenalectomized guinea-pig (31M) maintained on DOCA and injected daily for 22 days with 2·5 units of thyrotrophic hormone. The appearance is that of a resting gland. × 180.

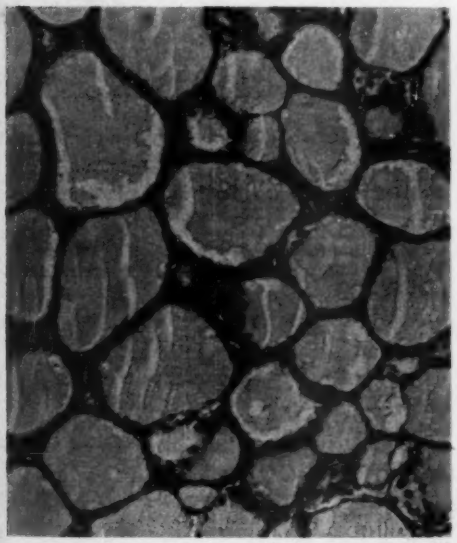


**Fig. 3.** Thyroid of normal guinea-pig (18M) injected daily for 22 days with 2·5 units of thyrotrophic hormone. The appearance is similar to those of Figs. 1 and 2, although the colloid has not stained as well. The epithelium is flat and the appearance is almost that of a resting gland. × 180.

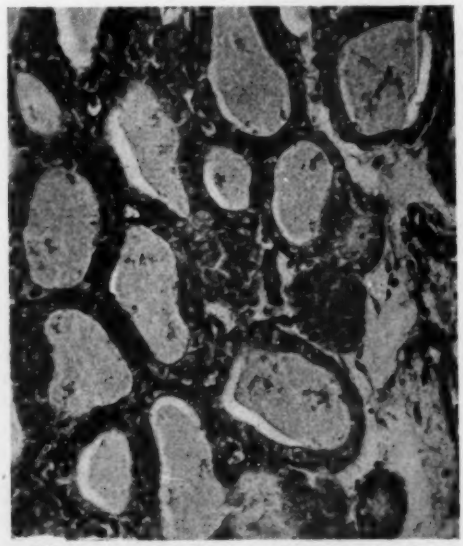


**Fig. 4.** Thyroid of normal guinea-pig (65F) injected daily for six days with 2·5 units of thyrotrophic hormone. The colloid is pale and vacuolated, the epithelium semi-columnar with commencing hyperplasia and early papillary infolding. The appearance contrasts markedly with those of Figs. 1, 2, and 3. × 180.

climatic conditions were not as suitable for the maintenance of adrenalectomized animals. Undoubtedly, increased operative shock due to a less expert technique was also a contributory factor. The low values for liver glycogen obtained in the later successful experiment reported in the case of adrenalectomized animals maintained on DOCA, whether subjected to thyrotrophic hormone injections or not, indicate the narrow margin by which these animals survive, and show how the superimposition of even a slightly unfavourable factor might precipitate a lethal result.

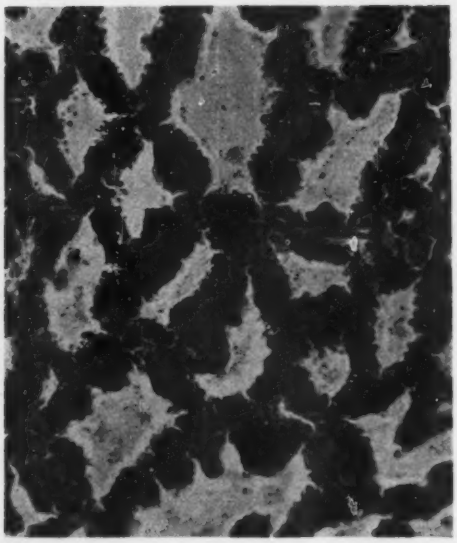


**Fig. 5.** Thyroid of adrenalectomized guinea-pig (251M) maintained on DOCA for 17 days and injected daily for 15 days with 20 units of thyrotrophic hormone. The colloid is pale and scanty, the epithelium columnar with marked papillary infolding and distorted acini. The appearance is that of an extremely active gland. × 180.

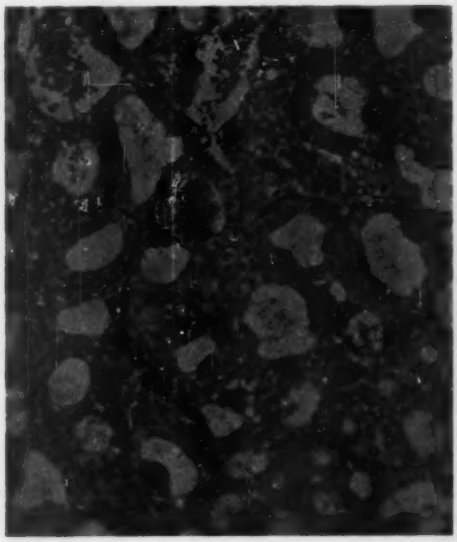


**Fig. 6.** Thyroid of a normal guinea-pig injected daily for 12 days with 20 units of thyrotrophic hormone (for comparison with Fig. 5). The gland was taken from an animal in another investigation running concurrently and in which the same batch of hormone and animals from the same stock were used. The appearance is one of intense activity, but not quite as marked as in Fig. 5. × 180.

## SUMMARY.

Resistance to thyrotrophic hormone injections, as shown by involution of the thyroid gland histologically following prolonged injections of small doses of thyrotrophic hormone, develops just as readily in adrenalectomized as in normal animals.

Adrenalectomized animals maintained on DOCA exhibit a profound fall in liver glycogen values, and the administration of thyrotrophic hormone to these animals reduces the liver glycogen after 14–15 days to even lower values.

The ascorbic acid content of the liver in adrenalectomized animals is significantly elevated after the administration of thyrotrophic hormone (for 14–15 days).

It is concluded that the adrenals play no rôle in the development of resistance to the administration of an heterologous preparation of the thyrotrophic hormone, and that the adrenalectomized animal responds in a similar manner to thyrotrophic hormone injections as does the normal animal.

*Acknowledgments*: We desire to record our thanks to Miss M. G. Crabtree for the liver glycogen assays, Mr. W. J. Ellis for assistance during the experiments, Miss N. McArthur, Haley Research Scholar, Hall Institute, Melbourne, for the statistical treatment of the results and Mr. S. Woodward-Smith for the photographs.

## REFERENCES.

Baumann, E. J. and Marine, D. (1945): Endocrin. 36, p. 400.
Birch, T. W., Harris, L. J. and Ray, S. N. (1933): Biochem. J., 27, p. 590.
Collip, J. B. and Anderson, E. M. (1934): Lancet, 226, p. 76.
Collip, J. B. (1935): J. Amer. med. Assoc., 104, p. 916.
Collip, J. B. (1938): Edinburgh med. J., 45, p. 782.
Crabtree, M. and Trikojus, V. M. (1946): Biochem. J., 40, p. 465.
Eitel, H. and Loeser, A. (1932): Arch. exp. Path. Pharmak., 167, p. 381.
Grattan, J. F. and Jensen, H. (1940): J. biol. Chem., 135, p. 511.
Grollman, A. (1936): The Adrenals (London: Bailliere, Tindall and Cox), pp. 143–6.
Hertz, S. and Kranes, A. (1934): Endocrin., 18, p. 415.
Junkmann, K. and Schoeller, W. (1932): Klin. Wschr., 11, p. 1,176.
Loeb, L. and Friedman, H. (1931): Proc. Soc. exp. Biol. Med., 29, p. 172.
Loeser, A. (1933): Arch. exp. Path. Pharmak., 173, p. 62.
Loeser, A. (1934): *Ibid.*, 176, p. 729.
Loeser, A. (1936a): Zbl. inn. Med., 57, p. 569.
Loeser, A. (1936b): Arch. exp. Path. Pharmak., 180, p. 325.
Loeser, A. and Trikojus, V. M. (1937): *Ibid.*, 185, p. 227.
Loeser, A., Ruland, H. and Trikojus, V. M. (1938): *Ibid.*, 189, p. 665.
Long, C. N., Katzin, B. and Fry, E. C. (1940): Endocrin., 26, p. 309.
McQueen-Williams, M. (1934): Proc. Soc. exp. Biol. Med., 32, p. 296.
McQuillan, M. T. and Trikojus, V. M. (1946): Brit. J. exp. Path. 27, p. 247.
Mahaux, J. (1938): Compt. rend. Soc. biol., 129, p. 39.
Mason, H. L. (1940): Proc. Staff Meet. Mayo Clinic, 15, p. 290.
Miller, D. S. (1938): Proc. Soc. exp. Biol. Med., 38, p. 453.
Olson, R. E., Thayer, S.A. and Kopp, L. J. (1944): Endocrin., 35, p. 464.
Perla, D. and Sandberg, M. (1937): Arch. Path., 23, p. 227.
Robinson, A. R. (1941): Austral. J. exp. Biol. 19, p. 261.
Schmeckebier, M. M. (1934): Proc. Soc. exp. Biol. Med., 31, p. 261.
Schockaert, J. A. (1932): Amer. J. Anat., 49, p. 379.
Siebert, W. J. and Smith, R. S. (1930): Amer. J. Physiol., 95, p. 396.
Silvette, H. and Britton, S. W. (1936): *Ibid.*, 116, p. 143.
Thompson, K. W. (1941): Physiol. Rev., 21, p. 588.
Trikojus, V. M. and Ellis, W. J. (1939): Austral. J. exp. Biol., 17 p. 441.
Watanabe, I. (1939): Tohoku J. exp. Med., 35, p. 65.
Wells, B. B. and Kendall, E. C. (1940a): Proc. Staff Meet. Mayo Clinic, 15, p. 133.
Wells, B. B. and Kendall, E. C. (1940b): *Ibid.*, 15, p. 565.
Wells, B. B. (1940): *Ibid.* 15, p. 294.
Werner, S. C. (1938): Endocrin., 22, p. 291.

# HISTAMINE AND HISTAMINASE IN CHRONIC MYELOID LEUKAEMIA OF MAN

## I. HISTAMINE IN THE BLOOD OF CHRONIC MYELOID LEUKAEMIA

by J. B. THIERSCH[1]

(From the Institute of Medical and Veterinary Science, Adelaide).

*(Accepted for publication 30th September, 1946.)*

Loos (1931) was the first to suggest that polymorphonuclear leucocytes contain histamine when he related an increased histamine content of inflammatory foci in the rabbit to the increased polymorphonuclear leucocyte count of that area. This observation was followed by the work of Code and Ing (1937) who isolated and identified histamine from the white cell layer of centrifuged rabbit blood. Code (1937) made an extensive study of the histamine content of the blood of rabbits, horses, bullocks, goats, dogs and man. He found that in these species the polymorphonuclear leucocytes contain most of the histamine of the blood. The author found an increased amount of histamine in sterile pleural exudates of the rabbits with an increased number of eosinophil leucocytes and in the blood of patients with eosinophilia due to parasites.

Code and Jensen (1941), comparing the histamine content of blood and bone marrow of guinea-pigs, rabbits, cats, dogs, a horse and a cow, found a definite increase in the bone marrow provided cellular red marrow was examined.

### *Histamine in the Blood of Man.*

Barsoum and Gaddum (1935) reported as normal 0·3–0·04γ histamine per c.c. of human blood. Haworth and MacDonald (1937) found an average of 0·01–0·8γ histamine per c.c. of blood in 103 apparently normal university students. Code (1937) gave 0·06γ histamine as normal value for human blood. Much higher contents, 0·18–1·0γ histamine per c.c. of whole blood, were reported by Code (1937), and Code and MacDonald (1937) in cases with parasitic infestation with an eosinophilia of the blood. Still larger quantities of histamine were found by Mahon, Parker and Corusa (1936), by Code and MacDonald (1937) and Went and Kiss (1941) in cases of myeloid leukaemia. Values found by these authors varied greatly, ranging from 0·1γ per c.c. to 15γ histamine per c.c. of whole blood. The histamine content was only roughly connected with the increased leucocyte count of the blood, and in one case combined with a high basophil cell count (Code). Code and MacDonald (1937) also reported a fall of the histamine content in cases of myeloid leukaemia after irradiation, coinciding with a fall in leucocytes.

### METHOD.

Venous blood was taken from patients suffering from myeloid leukaemia, with as little congestion of the veins as possible. Apart from the histamine estimation of whole blood with Code's method, a direct histamine estimation of the heparinised plasma was carried out. The blood was transferred at once into a container with heparin or citrate for the direct estima-

[1]Neale Research Pathologist to the Anti-Cancer Campaign Committee, University of Adelaide.

tion of the plasma histamine. For the histamine estimation on whole blood 10 c.c. of blood were transferred into 15 c.c. of 10 p.c. trichloracetic acid. The histamine was then extracted according to Code's modification of Barsoum and Gaddum's method. The histamine was finally assayed by measuring the contraction of a strip of jejunum of the guinea-pig in a small perfusion chamber. Control estimations of the heparin and citrate solutions gave no contractions.

TABLE 1.

*Histamine with Code's method in cases of chronic myeloid leukaemia.*

| Patient. | White blood count. | Total cell count of myeloid series. | Histamine in γ per ml. |
|---|---|---|---|
| 1 | 7,600 | 7,000 | 0·08 |
| 2 | 8,600 | 7,900 | 0·16 |
| 3 | 15,800 | 15,500 | 0·15 |
| 4 | 17,600 | 17,100 | 0·14 |
| 5 | 22,600 | 20,340 | 0·60 |
| 6 | 24,900 | 24,500 | 0·15 |
| 7 | 37,900 | 37,000 | 0·80 |
| 8 | 64,300 | 64,300 | 1·20 |
| 9 | 77,000 | 77,000 | 1·50 |
| 10 | 124,000 | 122,000 | 2·00 |
| 11 | 131,000 | 124.000 | 0·60 |
| 12 | 161,000 | 149,000 | 0·35 |
| 13 | 184,000 | 184,000 | 3·20 |
| 14 | 241,000 | 241,000 | 0·38 |
| 15 | 274,000 | 274,000 | 2·40 |
| 16 | 358,000 | 343,000 | 6·00 |

## RESULTS.

The values found in the blood of cases of chronic myeloid leukaemia ranged from 0·08γ per c.c. from a case with a normal leucocyte count to 6·0γ per c.c. of blood in a case with a leucocyte count of 358,000 per c.mm. Under deep X-ray treatment a steady decline of the leucocyte count and histamine content was noted, but no direct numerical relationship could be detected (see Table 1). The plasma of cases of chronic myeloid leukaemia contains only traces of histamine; the cellular elements of the blood contain practically all the histamine present in total blood.

## DISCUSSION.

According to Code and Ing (1937), Mahon, Parker and Corusa (1936), Code and MacDonald (1937), and Went and Kiss (1941), most of the histamine of the blood is found in the white blood cell layers of centrifuged blood. In rabbits Zon, Ceder and Crigler (1939) showed that the platelets and not the leucocytes were the main source of histamine in the blood. Their findings were not confirmed by other workers. Reid and Bick (1942) and Reid (1946) also found a substance in human platelets and serum which had a vasoconstrictor and smooth muscle stimulating activity. This substance was not extractable with ethyl alcohol, ether or methyl alcohol and it was definitely not regarded as histamine by the authors.

The findings of Mahon, Parker and Corusa, of Code and McDonald, and of Went and Kiss were confirmed by the investigations reported here.

## SUMMARY.

Histamine in the blood of cases of chronic myeloid leukaemia ranged from 0·08γ per cubic centimetre of blood with a normal leucocyte count to 6·0γ per c.c. of blood with a white blood count of 358,000.

Under deep X-ray treatment a steady decline of white blood count and histamine was noted.

The plasma of cases of chronic myeloid leukaemia contains only traces of histamine.

## II. HISTAMINE AND HISTAMINASE IN THE BLOOD OF CASES OF MYELOID LEUKAEMIA UNDER AND AFTER DEEP X-RAY TREATMENT.

Previous investigations by Code (1937), Code and Ing (1937), Code and MacDonald (1937), Mahon, Parker and Corusa (1936), Went and Kiss (1941), indicated that under normal conditions the leucocytes of the myeloid series carry histamine which comprises practically all the histamine in the circulating blood. In cases of myeloid leukaemia with a high leucocyte count the histamine rises approximately with the increased W.B.C., reaching 100 to 200 times normal values.

On examination it was found that while the leucocytes were intact the blood plasma contained little or no histamine; with the disintegration of the white cells a liberation of the histamine into the plasma could be expected. I thought it of interest to study the behaviour of histamine in patients with a high W.B.C. under deep X-ray treatment. The blood of cases of myeloid leukaemia shows a rapid fall in leucocyte count even after small doses of deep X-ray to the spleen. A fall in W.B.C. of 50 to 100,000 per c.mm. within 48 hours following irradiation is frequent; falls of 2–300,000 within a week or a fortnight are usual. As the rate of the production of leucocytes is unknown, it has been open to discussion whether irradiation effected an increased destruction or a slowing down of the production of these elements. If deep X-ray treatment produced an increased rate of destruction of leucocytes an increased amount of histamine in the circulating blood plasma could be expected.

### METHODS.

Patients suffering from chronic myeloid leukaemia with an enlargement of the spleen and increased W.B.C. were given a course of deep X-ray treatment. Usually the patients received a course of treatment with deep X-rays to the enlarged spleen in doses of 100 AP and 100 PA, making a total of 500–600r given on alternate days. Some days before and, on days of irradiation, immediately following the exposure to deep X-rays, blood was carefully taken from the cubital vein in heparin or citrate. Blood was also collected some days after the irradiation when the W.B.C. had been markedly reduced. The plasma was separated and its histamine content examined. Control investigations of the heparin and citrate solutions gave no contractions of the gut.

### RESULTS.

The investigations showed that no increased amount of histamine liberated from the leucocytes was found in the plasma (Table 2). The lack of a rise of histamine in the plasma might have been due to an increase in histaminase reducing the liberated histamine in the circulating blood. This is known in pregnancy where the enzyme shows a marked increase in the plasma. The presence and activity of this enzyme are estimated by adding a known quantity of histamine to the plasma and by measuring quantitatively the amount of histamine still present after several hours of incubation. The histaminase was measured by adding 0·1 c.c. of 1:100,000 histamine phosphate (B.D.H.) to 1·9 c.c. of plasma, producing a minimal dilution of 1:2,000,000 per c.c. of histamine. This mixture was incubated for 5–6 hours in a water bath at 37° C. with a carbogen atmosphere (5 p.c. $CO_2$ in oxygen) in order to maintain the pH. Plasmas from pregnant women (Ahlmark, 1944) served as controls which in all cases digested totally the added histamine in half of the incubation period. In cases of chronic myeloid leukaemia with or without treatment, with and without irradiation, no appreciably increased amount of histaminase could be detected with a period of observation of 5–6 hours (Table 2).

### DISCUSSION.

According to the findings, irradiation slows down the process of production of the leukaemic cells, either directly by inhibiting their output from the leukaemic foci in the body or by restoring an inhibitory influence of the spleen on the leukaemic production.

Irradiation does, however, not effect a breakdown of leukaemic elements which might necessitate the presence of more enzyme to avoid severe reactions from the liberated histamine.

TABLE 2.

*Histamine in myeloid leukaemia.*

| Patient. | Irradiation. Date of irradiation. | Date of assay. | Total white blood cell count. | Total cell count of myeloid series. | Plasma histamine in γ per ml. | Plasma histaminase—expressed as γ histamine per ml. after digestion for 5–6 hours. | |
|---|---|---|---|---|---|---|---|
| McL. | | 19/7/45 | 14,700 | 13,200 | ·03 | ·08 | 5 hrs. |
| Cl. | | 25/7/45 | 19,000 | 17,850 | Nil | ·03 | 5 hrs. |
| Ro. | | 28/6/45 | 91,000 | 91,000 | ·06 | Nil | 5 hrs. |
| Ri. | | 6/7/45 | 750,000 | 750,000 | ·02 | ·03 | 5 hrs. |
| Brt. | 100r 11/7/45 | 11/7/45 | 586,000 | 586,000 | ·07 | ·05 | 6 hrs. |
| | 100r 13/7/45 | | | | | | |
| | 50r 17/7/45 | 17/7/45 | 253,000 | 253,000 | ·03 | ·07 | 5 hrs. |
| | 50r 19/7/45 | | | | | | |
| | 50r 23/7/45 | | | | | | |
| | 50r 26/7/45 | 26/7/45 | 81,000 | 80,300 | ·03 | ·01 | 5 hrs. |
| | 50r 30/7/45 | | | | | | |
| | 50r 2/8/45 | | | | | | |
| | 70r 6/8/45 | | | | | | |
| | 70r 8/8/45 | | | | | | |
| | 70r 10/8/45 | | | | | | |
| | 15 days after | 25/10/45 | 22,600 | 20,340 | Nil | ·04 | 6 hrs. |
| Ea. | 5 days before | 19/7/45 | 45,500 | 43,000 | ·02 | ·06 | 5 hrs. |
| | 100r 24/7/45 | | | | | | |
| | 100r 25/7/45 | 25/7/45 | 90,000 | 90,000 | Nil | ·02 | 5 hrs. |
| | 100r 26/7/45 | | | | | | |
| | 100r 27/7/45 | 27/7/45 | 42,000 | 42,000 | ·01 | ·01 | 5 hrs. |
| | 125r 30/7/45 | | | | | | |
| | 125r 31/7/45 | 31/7/45 | 40,000 | 40,000 | ·02 | ·03 | 5 hrs. |
| McR. | 6 days before | 17/10/45 | 133,000 | 133,000 | Nil | ·03 | 5 hrs. |
| | 150r 23/10/45 | 23/10/45 | 86,000 | 86,000 | Nil | Nil | 5 hrs. |
| | 150r 25/10/45 | 25/10/45 | 78,000 | 77,000 | Nil | Nil | 5 hrs. |
| | 150r 27/10/45 | | | | | | |
| | 150r 29/10/45 | 29/10/45 | 41,000 | 39,000 | Nil | ·02 | 5 hrs. |
| | 9 days after | 7/11/45 | 9,000 | 8,500 | ·04 | Nil | 5 hrs. |
| | 37 days after | 5/12/45 | 10,300 | 8,000 | Nil | Nil | 5 hrs. |
| Wi. | 3 days before | 27/8/45 | 161,000 | 149,200 | Nil | ·01 | 6 hrs. |
| | day before | 29/8/45 | 161,000 | 149,200 | ·02 | Nil | 5 hrs. |
| | 100r 30/8/45 | | | | | | |
| | 100r 31/8/45 | 31/8/45 | 241,000 | 241,000 | ·08 | ·04 | 5 hrs. |
| | 100r 3/9/45 | | | | | | |
| | 100r 4/9/45 | 4/9/45 | 200,000 | 196,000 | ·07 | ·07 | 6 hrs. |
| | 100r 5/9/45 | | | | | | |
| | 100r 6/9/45 | | | | | | |
| | 120r 7/9/45 | 7/9/45 | 82,000 | 82,000 | ·03 | ·04 | 6 hrs. |
| | 120r 10/9/45 | | | | | | |
| | 2 days after | 11/9/45 | 8,000 | 6,710 | ·01 | ·03 | 6 hrs. |
| | 3 days after | 13/9/45 | 7,700 | 7,200 | ·02 | Nil | 6 hrs. |
| Tr. | 110 days before | 28/6/45 | 6,000 | 5,030 | ·04 | Nil | 2 hrs. |
| | 83 days before | 25/7/45 | 66,000 | 66,000 | Nil | ·02 | 6 hrs. |
| | 6 days before | 17/10/45 | 185,000 | 177,000 | Nil | ·01 | 6 hrs. |
| | 200r 23/10/45 | 23/10/45 | 183,000 | 179,000 | Nil | Nil | 6 hrs. |
| | 200r 25/10/45 | 25/10/45 | 141,000 | 138,000 | Nil | ·03 | 6 hrs. |
| | 200r 29/10/45 | 29/10/45 | 68,000 | 66,000 | Nil | Nil | 6 hrs. |
| Hei. | 8 days before | 31/10/45 | 345,000 | 340,000 | Nil | Nil | 6 hrs. |
| | 230r 7/11/45 | 7/11/45 | 274,000 | 274,000 | ·06 | ·06 | 6 hrs. |
| | 230r 9/11/45 | 9/11/45 | 184,000 | 184,000 | ·01 | ·01 | 6 hrs. |
| Ri. | 142 days before | 13/7/45 | 190,000 | 186,000 | Nil | ·05 | 5 hrs. |
| | 100r 3/12/45 | 3/12/45 | 478,000 | 478,000 | Nil | Nil | 6 hrs. |
| | 100r 5/12/45 | 5/12/45 | 446,000 | 446,000 | Nil | Nil | 6 hrs. |
| | 100r 7/12/45 | 7/12/45 | 274,000 | 274,000 | Nil | Nil | 6 hrs. |

SUMMARY.

The histamine content of blood plasma of cases of myeloid leukaemia is not higher than that of plasma of normal people.

The histamine content of the plasma does not increase under and after irradiation with deep X-rays when the high W.B.C. falls.

No increase in histaminase was found in cases of myeloid leukaemia without and under treatment with deep X-ray.

The investigations indicate that irradiation impedes the output of leukaemic cells but does not directly destroy the cells on a large scale.

*Acknowledgment.* I wish to thank Professor E. R. Trethewie for his help in this work.

REFERENCES.

Ahlmark, A. (1944): Lancet 246, p. 408.
Barsoum, G. S. and Gaddum, J. H. (1935): J. Physiol., 81, p. 1.
Code, C. F. (1937): J. Physiol., 89, p. 256.
Code, C. F. (1937): *Ibid.*, 90, p. 349.
Code, C. F. (1937): *Ibid.*, 90, p. 485.
Code, C. F. and Ing, H. R. (1937): *Ibid*, 90, p. 501.
Code, C. F. and Jensen, J. L. (1941): Amer. J. Physiol., 131, p. 768.
Code, C. F. and MacDonald, A. D. (1937): Lancet, 223, p. 730.
Loos, H. O. (1931): Arch. f. Dermat. u. Syph., 164. p. 199.
Mahon, I, Parker, C. C. and Corusa, G. (1936): Bull. Soc. Med. Hosp. Budapest, 18, p. 246.
Reid, G. and Bick. M. (1942): Austral. J. exp. Biol., 20, p. 33.
Reid, G., (1946): *Ibid.*, 24, p. 327.
Went, F. and Kiss, B. R. (1941): Z. klin. Med., 139, p. 157.
Zon, L., Ceder, E. T. and Crigler, C. W. (1939): Pub. Health Rep. 54, p. 1,978.

# HISTAMINE AND HISTAMINASE IN THE BLOOD OF CASES OF LYMPHOID AND MONOCYTIC LEUKAEMIA

by J. B. THIERSCH[1]

(From the Institute of Medical and Veterinary Science, Adelaide).

*(Accepted for publication 30th September, 1946.)*

Code (1937) failed to detect quantities of histamine in the lymph nodule of the rabbit and horse. Code and MacDonald (1937) found normal blood values ranging from 0·021γ to 0·086γ in cases of lymphoid leukaemia with high lymphocyte counts in the blood varying from 117,000 to 413,000 per c.mm. Went and Kiss (1941) also reported normal histamine blood contents in cases of lymphoid leukaemia. Code (1937) found no increase of histamine in an experimentally produced monocytic pleural exudate of the rabbit.

## EXAMINATIONS.

In the blood of 6 cases of chronic lymphoid leukaemia Code's modification of Barsoum and Gaddum's technique gave the same results as the above-mentioned authors (see Table 1). In no case was more than 0·08γ of histamine per c.c. found in whole blood. The total lymphocyte count varied from 336,000 to 7,000 per c.mm. The platelets were normal in numbers, or scanty.

The histamine and histaminase of the plasma of cases of chronic lymphoid leukaemia were examined (Table 1) as described previously (Thiersch, 1947). In three cases the plasma of untreated patients was investigated. In two further cases with gross enlargement of the spleen and lymph nodules and a high W.B.C. a course of deep X-ray was followed with plasma histamine and histaminase estimations. The plasma was collected in these two cases the day before onset of treatment and from then on after every single exposure to deep X-ray until the full course was completed. The irradiation in both cases consisted of 50r PA and AP to the spleen per dose with a total of 1,000r in the first case and a total of 700r in the second case.

## DISCUSSION.

The low histamine content of the blood of cases of chronic lymphoid and monocytic leukaemias suggests a common origin of the lymphocyte and monocyte.

## SUMMARY.

The blood content of histamine in cases of chronic lymphoid leukaemia in man is normal. This result confirms the work of other workers.

Under deep X-ray treatment no increased amount of histamine or histaminase was found in cases of lymphoid leukaemia.

The histamine content of the blood, spleen and lymph nodules of monocytic leukaemias was found to be normal with Code's method.

The histamine and histaminase content of the plasma of a case of a typical monocytic leukaemia was found to be normal.

*Acknowledgment.* I wish to thank Professor E. R. Trethewie for his help in this work.

## REFERENCES.

Barsoum, G. S. and Gaddum, J. H. (1935): J. Physiol., 81, p. 1.
Code, C. F. (1937): *Ibid.*, 90, p. 485.
Code, C. F. (1937): *Ibid.*, 89, p. 256.
Code, C. F. and MacDonald, A. D. (1937): Lancet, 223, p. 730.
Zon, L. Ceder, E. T. and Crigler, C. W. (1939): Pub. Health Rep., 54, p. 1,978.
Thiersch, J. B. (1947): Austral. J. exp. Biol., 25, p. 73.

[1]Neale Research Pathologist to the Anti-Cancer Campaign Committee, University of Adelaide.

TABLE 1.

*Histamine and histaminase in lymphatic leukaemia.*

| Name. | | Date. | Method. | W.B.C. | Total polymorphs. | Histamine in γ per c.c. | Plasma histaminase expressed as γ histamine per ml. after digestion for 5–6 hours. | |
|---|---|---|---|---|---|---|---|---|
| Br. | | 8/8/45 | Direct | 600,000 | — | ·02 | ·12 | 5 hrs. |
| | | 21/8/45 | Direct | 330,000 | — | ·08 | ·11 | 5 hrs. |
| | | 27/8/45 | Direct | 340,000 | — | Nil | ·12 | 6 hrs. |
| | | 29/8/45 | Direct | 340,000 | 3,400 | Nil | ·12 | 5 hrs. |
| | post irradiation | | | | | | | |
| | 200r | 7/9/45 | Direct | 414,000 | 8,280 | ·04 | ·12 | 6 hrs. |
| | 200r | 11/9/45 | Direct | 500,000 | — | ·02 | ·14 | 6 hrs. |
| 1,000r | 200r | 13/9/45 | Direct | 363,000 | — | ·02 | ·12 | 6 hrs. |
| | 200r | 23/9/45 | Code | 300,000 | 1,500 | Nil | | |
| | 200r | 29/9/45 | Code | 340,000 | 3,400 | ·02 | | |
| | | 9/10/45 | Direct | 524,000 | — | Nil | ·10 | 6 hrs. |
| | | 12/10/45 | Direct | 600,000 | — | — | No histaminase | |
| | | 15/10/45 | Direct | 330,000 | — | ·01 | ·10 | 6 hrs. |
| | | 17/10/45 | Direct | 299,000 | — | Nil | ·10 | 6 hrs. |
| | | 19/10/45 | Direct | 360,000 | — | Nil | ·10 | |
| | P.M. | 23/10/45 | Code | Blood | | ·10 | | |
| | | | Code | Spleen | | ·35 | | |
| | | | Code | Lymph Nodules | | ·26 | | |
| | | | Code | Clot | | ·08–·19 | | |
| Fe. | | 31/5/45 | Code | 13,300 | 7,200 | ·07 | | |
| | no irradiation | 31/8/45 | Direct | 97,000 | 3,876 | ·05 | ·16 | 5½ hrs. |
| | | 31/8/45 | Code | 97,000 | 3,870 | ·20 | | |
| | | 1/11/45 | Code | 58,000 | 1,000 | ·02 | | |
| Ka. | day after irradiation | | | | | | | |
| | 100r | 21/8/45 | Direct | 40,000 | — | ·08 | ·06 | 5 hrs. |
| | 100r | 23/8/45 | Code | 30,600 | 1,820 | Nil | | |
| | | 1/11/45 | Code | 20,900 | 1,000 | ·01 | | |
| Le. | | | | | | | | |
| | no irradiation | 5/7/45 | Direct | 62,000 | 2,070 | ·07 | ·08 | 5 hrs. |
| | | | Code | 62,000 | 2,070 | ·07 | | |
| Pa. | | | | | | | | |
| | no irradiation | 29/8/45 | Direct | 189,000 | 9,430 | ·04 | ·05 | 5 hrs. |
| | | 29/8/45 | Code | 189,000 | 9,430 | ·02 | | |
| | 150r | 12/10/45 | Direct | 182,000 | 10,900 | Nil | ·10 | 6 hrs. |
| 600r | 150r | 15/10/45 | Direct | 169,000 | 10,900 | Nil | ·10 | 6 hrs. |
| | 150r | 17/10/45 | Direct | 62,000 | 3,820 | Nil | ·10 | 6 hrs. |
| | 150r | 19/10/45 | Direct | 30,000 | 4,670 | ·05 | ·12 | 6 hrs. |
| 4 days after | | 23/10/45 | Direct | 6,900 | 2,200 | ·01 | ·11 | 6 hrs. |
| 6 days irrad- | | 25/10/45 | Direct | 6,900 | 4,140 | Nil | ·09 | 6 hrs. |
| 10 days iation | | 29/10/45 | Direct | 6,300 | 3,150 | Nil | ·12 | 6 hrs. |

On direct examination, with Code's method, of whole blood, spleen and lymph nodules from 3 cases of typical monocytic leukaemia no increased amount of histamine was detected (see Table 2). The plasma histamine was also not increased in one case before and under deep *X*-ray therapy (see Table 2). The histaminase was found to be slightly higher than normal under deep *X*-ray therapy, but only a fraction of the amount found in pregnancy.

TABLE 2.

*Histamine in monocytic leukaemia.*

| Name | Date. | Method. | W.B.C. | Total polymorphs. | Total lymphocytes. | Total monocytes. | Histamine in γ per c.c. or per gm. tissue. | Plasma histaminase expressed as γ histamine per ml. after digestion for 5–6 hours. | |
|---|---|---|---|---|---|---|---|---|---|
| Wi. before irradiation | 28/6/45 | Direct | 17,100 | 9,600 | 2,900 | 4,600 | ·03 | nil | 6 hrs. |
| before irradiation | 3/8/45 | Direct | 61,500 | 39,000 | 5,000 | 17,500 | ·09 | 0·10 | 6 hrs. |
| before irradiation | 31/8/45 | Direct | 64,000 | 45,000 | 5,000 | 14,000 | ·02 | 0·11 | 6 hrs. |
| total 125γ | 4/9/45 | Direct | 73,000 | 52,000 | 4,500 | 16,000 | ·09 | 0·12 | 6 hrs. |
| total 250γ | 7/9/45 | Direct | 71,000 | 49,500 | 4,500 | 17,000 | ·07 | 0·11 | 6 hrs. |
| total 500γ | 11/9/45 | Direct | 20,500 | 12,550 | 1,450 | 6,500 | ·01 | 0·11 | 6 hrs. |
| total 750γ | 13/9/45 | Direct | 28,300 | 19,500 | 850 | 8,000 | ·01 | 0·10 | 6 hrs. |
| | 31/8/45 | Code | 64,000 | 45,000 | 5,000 | 17,500 | ·06 | | |
| Wi. P.M. | 14/10/45 | | | | | | | | |
| | Blood | Code | 99,000 | 79,000 | 5,000 | 15,000 | ·03 | | |
| | Spleen | Code | No cell counts possible | | | | ·03 | | |
| | Clot | Code | No cell counts possible. | | | | ·03 | | |
| Ti. | 19/9/45 Sternal Puncture | Code | 17,900 | 13,210 | 1,610 | 718 | Nil | | |
| Lo. | 22/5/46 | Direct | 233,000 | 228,000 | 400 | 4,600 | ·07 | | |
| | | Code | 233,000 | 228,000 | 400 | 4,600 | ·04 | | |
| Lo. P.M. | 24/5/46 | | | | | | | | |
| | Blood | Code | 181,000 | 177,000 | 400 | 3,600 | ·10 | | |
| | Lymph Nodule | Code | No cell counts possible. | | | | ·10 | | |
| | Lymph Nodule | Code | | | | | ·04 | | |

# TOXICITIES OF THE OPTICAL ISOMERS OF NICOTINE AND NORNICOTINE

by C. STANTON HICKS AND D. A. SINCLAIR[1]

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

*(Accepted for publication 7th October, 1946.)*

A preliminary test of the toxicity of d-nornicotine to rats was made by Hicks and LeMessurier (1935), who used the partially racemized alkaloid obtained from *Duboisia Hopwoodii*. They found it to be about three times as toxic as l-nicotine. We have made a more exact and more comprehensive study of the toxicities of the optical isomers of both nicotine and nornicotine to rats and guinea-pigs and have also made an attempt to assess the insecticidal powers of d-nornicotine compared with that of l-nicotine, using mosquito larvae as test insects.

## PREPARATION OF COMPOUNDS.

*d-Nornicotine.* The naturally occurring alkaloid is racemized to the extent of about 50 p.c. The dextro form can be separated from the racemic by fractional crystallization of the diperchlorate from methyl alcohol plus ether as described by Späth (1935). It is important to have dry solvents as a small amount of water present leads to the precipitation of an oil instead of crystals on addition of the ether. In such cases the admixture of acetone was found to favour crystallization, but unfortunately, also seems to prevent the separation of the dextro-alkaloid from the racemic. The highest specific rotation obtained was $[\alpha]_D = 84{\cdot}0°$.

*l-Nornicotine.* We obtained a small quantity of l-nornicotine by the method described by Späth (1936), viz., oxidation of l-nicotine with silver oxide. The yield is very small, about 2 p.c. The specific rotation of the product was $[\alpha]_D = -82{\cdot}6°$. A further supply was obtained from the alkaloids of *Nicotiana Excelsior* by fractional extraction.

*l-Nicotine.* Commercial nicotine purified by distillation was used. The specific rotation was $[\alpha]_D = -166°$.

*d-Nicotine.* This we obtained from our d-nornicotine by methylation with formaldehyde plus formic acid (see Späth, 1935). The yield was theoretical and the product of a high degree of optical purity — $[\alpha]_D = 168°$, thus confirming the purity of our d-nornicotine.

## TOXICITY EXPERIMENTS.

*Rats.* The monohydrochlorides of the alkaloids were always used, in solutions made up to have the same total molality as normal saline by the addition of the requisite amount of sodium chloride. This practice was adopted after preliminary experiments had revealed that considerable variation in toxicity could be brought about by varying the concentration of the injected solution whilst keeping the dose constant, due apparently to varying the rate of entry of the drug into the blood stream. Groups of ten animals were injected intraperitoneally with doses ranging about the medium lethal dose and characteristic toxicity curves were constructed by plotting number of deaths against dose expressed as the logarithm of the number of milligrams of alkaloid per kg. of rat. It is better to use the logarithmic scale as this makes the curves the same shape in all regions, whereas plotting against numerical dose gives flatter curves the higher the dose. Trevan (1927) considers that for regular results ten in a group is too small a number, and recommends thirty as being the lowest number that could reasonably be expected to give a smooth curve. However, for our purposes we found the results sufficiently regular and although points did occasionally fall well off the smooth curve, the customary shape of the curve being known, its position could be estimated with reasonable accuracy. For a more refined comparison of two approximately equal poisons, larger number of animals would certainly be necessary. We carried out the experiments on d-nicotine some months later than those on l-nicotine and found a difference of about 15 p.c. between the two curves; but on repeating two

[1]Under grant from the Council for Scientific and Industrial Research.

points with l-nicotine we found that these fell on the d-nicotine curve, indicating that the displacement of the curve was due to a change in the susceptibility of the rats. One series was carried out in winter and the other in summer, and the change in susceptibility was probably due to the temperature or some allied factor.

TABLE 1.

*Number of deaths in each group of ten animals injected with alkaloid dose expressed in milligrams per kg. of animal.*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***Rats.*** | | | | | | | | | |
| d-Nornicotine | Dose (mg./kg.) | 3 | 5 | 6 | 6·5 | 7 | | | |
| | Deaths | 0 | 2 | 4 | 8 | 10 | | | |
| d-Nornicotine (50 p.c. racemic) | Dose | 4·7 | 5·3 | 5·7 | 6·4 | 8·5 | | | |
| | Deaths | 0 | 2 | 4 | 9 | 10 | | | |
| dl-Nornicotine | Dose | 7·5 | 9 | 11 | | | | | |
| | Deaths | 1 | 1 | 5 | | | | | |
| l-Nornicotine | Dose | 15 | 20 | 25 | 29·6 | 35 | 45 | | |
| | Deaths | 0 | 3 | 8 | 7 | 10 | 10 | | |
| l-Nicotine (winter) | Dose | 16·5 | 18 | 20 | 22 | 25 | | | |
| | Deaths | 0 | 1 | 7 | 8 | 10 | | | |
| l-Nicotine (summer) | Dose | 20 | 23 | | | | | | |
| | Deaths | 0 | 4 | | | | | | |
| d-Nicotine (summer | Dose | 20 | 20 | 22·5 | 23 | 25 | 30 | 40 | 50 |
| | Deaths | 2 | 1 | 4 | 5 | 8 | 10 | 9 | 9 |
| ***Guinea-pigs.*** | | | | | | | | | |
| d-Nornicotine | Dose | 8 | 10 | 12 | 15 | | | | |
| | Deaths | 1 | 8 | 9 | 8 | | | | |
| l-Nornicotine | Dose | 17·5 | 30 | | | | | | |
| | Deaths | 1 | 6 | | | | | | |
| l-Nicotine | Dose | 30 | 35 | 35 | | | | | |
| | Deaths | 2 | 9 | 10 | | | | | |
| d-Nicotine | Dose | 30 | 35 | 35 | 40 | 50 | 62·5 | 75 | |
| | Deaths | 1 | 2 | 9 | 9 | 10 | 9 | 9 | |

Our results yield the surprising conclusion that whereas d-nornicotine is 3½ times as toxic as l-nornicotine there is no difference between the two nicotines. One would have expected a similar difference between the pairs of isomers and the fact that there is not, illustrates how complex is the problem of elucidating the mode of action of these drugs. Moreover, unpublished work reveals a marked difference between the nicotine isomers in their action on individual nerve centres such as the cervical ganglion of cats.

Within the limits of experimental error the toxicity of l-nornicotine appears to be identical with that of the nicotines. Also there is no detectable difference in toxicity between the pure dextro and the 50 p.c. racemized nornicotine. The 100 p.c. racemic, however, yields a curve at about the theoretical position between dextro and laevo.

Our findings are in disagreement with the general belief that d-nicotine is only half as toxic as l-nicotine, on the evidence of Mayor (1904) who, however, based his judgment on the results of injections into only one or two animals (guinea-pigs and rabbits), and individual cases can be very misleading. It was possible that there might be a difference between rats and guinea-pigs, which would account for the disagreement. To check this we carried out a similar series of experiments with the latter animals.

TABLE 2.

*Median lethal doses in milligrams per kg. of animal, estimated from curves in Figs 1 and 2.*

| | Rats. | | Guinea-pigs. |
|---|---|---|---|
| Alkaloid. | Season. | M.L.D. | (all in winter). |
| d-Nornicotine | Winter | 6 | 10 |
| l-Nornicotine | Summer | 23½ | 28 |
| dl-Nornicotine | Winter | 10½ | |
| l-Nicotine | Winter | 20 | 32 |
| l-Nicotine | Summer | 23½ | |
| d-Nicotine | Summer | 23½ | 33 |

*Guinea-pigs.* The same technique was adopted as with rats. Owing to the heterogeneity of the stock which was purchased from dealers, the results display somewhat greater irregularity than those with rats. Nevertheless, they are sufficiently clear to show that although these animals are more resistant than rats, the relative positions of the curves are about the same. It is possible that d-nicotine is very slightly less toxic than l-nicotine, but within the limits of experimental error they are the same. Only two points were determined for l-nornicotine which may be slightly superior in toxicity to the nicotines, while d-nornicotine is about 3 times as toxic. Trials with doses of d-nicotine much higher than the median lethal dose showed that a 1 in 10 chance of recovery persisted to as high as twice the median lethal dose. This chance may be due to the chance of the injection gaining access to a larger area of visceral peritoneum.

The chief difference in symptoms noticeable between the drugs was that the nicotines were much more rapid in action than the nornicotines. With a lethal dose of nicotine respiration would cease within ½ to 1 minute after injection followed by violent convulsions, the body being generally stretched out with back straight or concave. Death would ensue in 3 to 10 minutes. In the case of the nornicotines it was some minutes before convulsive fits set in, and these were different in appearance from those of the nicotine-injected animals, consisting of spasmodic jumps, the back remaining convexly arched. Death generally occurred in 6 to 14 minutes.

FIG. 1
CHARACTERISTIC TOXICITY CURVES OF RATS

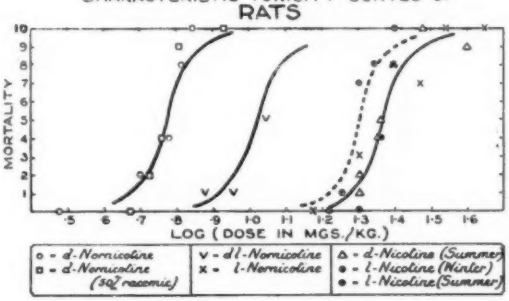
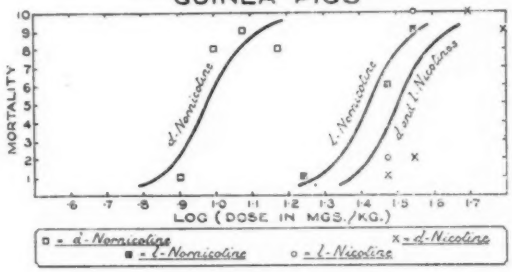



FIG. 2
CHARACTERISTIC TOXICITY CURVES OF GUINEA PIGS




*Mosquito Larvae.*

In view of the widespread use of nicotine as an insecticide, it is of interest to compare the toxicity to insects of nornicotine. Racemic nornicotine has already been tested on *Aphis rumicus* by Richardson, Craig, and Hansberry (1936), who found it to be equal with l-nicotine in toxicity. We made attempts to secure aphides from the Waite Institute, but owing to the depredation of parasites were unable to secure a satisfactory colony. We then turned our attention to mosquito larvae as possible test insects. A salt water species, *Aëdes concolor* Taylor, found in rock pools in New South Wales, seemed suitable, as the adults do not require blood and the larvae were shown by Woodhill (1936) to be capable of adapting themselves to salt concentrations varying from that of Sydney tap-water to several times that of sea water. Adelaide tap-water proved to be unsuitable; but a healthy colony was successfully reared in sea water at 25° C. Preliminary experiments indicated that these salt water larvae were much more resistant to nicotine than the fresh water species experimented with by Campbell, Sullivan and Smith (1933), who found a median lethal concentration of the order of 1 part in 10,000. We found that this concentration had little observable effect and that even with concentrations twenty times this, some still survived next day.

A series of beakers each containing about 50 larvae in 100 c.c. of sea water were treated with the neutral hydrochlorides of d-nicotine and d-nornicotine in amounts ranging from 1 in 250 to 1 in 10,000 of the alkaloids. They were set aside in an incubator at 25° C. until next day. Even in the 1 in 250 solutions some larvae still exhibited tremulous movements, though very feeble in character. Proceeding down the series to the less concentrated solutions, one observed gradually increasing mobility until at 1 in 10,000 no noticeable effects were apparent. In the intermediate solutions some larvae were at the surface with heads up (normally they hang with tails up), while others remained for long periods with heads down at the bottom of the beaker. Numbers of deaths were exceedingly difficult to count, as some larvae remained still for a long time, and though apparently dead, would respond to disturbance with a

needle. Even this was not a satisfactory guide, as some would not respond to slight disturbance but would wriggle on being more violently disturbed, and the dividing line between dead and alive was so vague that the attempt to count deaths was abandoned. In any case the characteristic curves would be so flat that accurate comparison would have been very difficult. General observation indicated that the toxicity of the nornicotine was of the same order as that of nicotine. If anything, it was slightly less toxic but certainly more than half as potent. It was noted that the insects possessed a reflex which caused them to swim against the current and when the water was stirred they tended to remain in the same position. Doubtless this reflex serves to preserve them from being washed out to sea in the natural state. It provided a useful test of the degree of partial paralysis, which begins to be observed in the 1 in 5,000 solution. There were 4 or 5 in the nicotines and 12 in the nornicotine still retaining the reflex. At 1 in 2,000 the reflex is completely gone, but about 10 p.c. of the larvae still seem capable of swimming more or less normally. At 1 in 1,000 no normal swimming is observed—only spasmodic movements and tremors; at 1 in 500 tremors and wriggling more feeble, and at 1 in 250 very feeble and only in one or two cases.

Larvae which had been in the 1 in 250 solutions for 22 hours and others which had been in the 1 in 500 solutions for two days were transferred to fresh sea water. On the following day there were observed about a dozen in each beaker fully recovered. The reason for the amazing resistance of these insects might well form the topic of further research. It is probably associated in some way with their capacity to adapt themselves to wide ranges of salt concentration.

*Acknowledgment.* We are indebted to Mr. D. C. Swan, of the Waite Institute, who reared the mosquito larvae for us.

## SUMMARY.

The relative toxicities of the optical isomers of nicotine and nornicotine to rats and guinea-pigs injected intraperitoneally have been determined, showing threefold difference between d-nornicotine and the others (equal). The larvae of the salt water mosquito *Aëdes concolor* show remarkable resistance to nicotine and nornicotine.

## REFERENCES.

Campbell, F. L., Sullivan, W. N. and Smith, C. R. (1933): J. econ. Entomol., 26, p. 500.
Mayor (1904): Arch. Sci. phys. et nat., 17, p. 418.
Richardson, C. H., Craig, L. C. and Hansberry, T. R. (1936): J. econ. Entomol., 29, p. 850.
Späth, E. and Zajic, E. (1935): Ber., 68, p. 1667.
Späth, E., Marion, L. and Zajic, E. (1936): *Ibid.*, 69, p. 251.
Trevan, J. W. (1927): Proc. Roy. Soc., Biol., p. 483.
Woodhill, A. R. (1936): Bull. entomol. Res., 27, p. 633.

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# THE USE OF STAINED SMEARS FOR THE RAPID DIAGNOSIS OF INFECTION WITH *STR. HAEMOLYTICUS* GROUP A

by HILDRED M. BUTLER

(From the Department of Pathology, the Women's Hospital, Melbourne).

*(Accepted for publication 12th March, 1947.)*

The great advances made in chemotherapy in the last ten years have increased the necessity for determining immediately the bacteria responsible for individual cases of infection.

In this laboratory we consider the development of methods for the rapid identification of bacteria to be a problem of prime importance, and whenever possible we use appropriately stained smears for this purpose. Our preliminary experience with puerperal and abortional infections showed that an immediate bacteriological diagnosis could be made in approximately three-quarters of the infections caused by *Str. haemolyticus* Group A. This work was based on the detection of capsulated cocci in Leishman-stained vaginal or cervical smears (Butler, 1946).

This success encouraged us to investigate the possibility of using direct smears for the diagnosis of Group A infections in general.

## MATERIAL STUDIED AND METHODS USED.

The specimens studied consisted of 1,230 throat swabs, 836 specimens from puerperal and abortional infections (this included the 554 previously reported) and 100 from patients with infected post-operative wounds, peritonitis, breast abscess, cellulitis, infections of the ear, joints, etc.

The throat swabs were conveniently divided into three groups depending on the source of the specimens. The first consisted of specimens from 376 cases of clinical throat infection among R.A.A.F. personnel. Cultures on horse blood agar plates and two unstained smears made from the throat swab were received from each patient.

The second group was composed of swabs from 617 children attending the Children's Hospital, Melbourne. These swabs were from children with sore throats, those suffering or convalescent from rheumatic fever, and from contacts or suspected carriers. Smears and cultures on horse blood agar plates were made from the swabs on arrival at this laboratory.

The third group consisted of: (a) swabs from 40 cases of sore throat occuring in this hospital and, (b) 197 swabs taken from apparently healthy nurses and students prior to commencing their work in the midwifery wards. Smears and cultures on horse blood agar plates were made from the swabs immediately they were obtained.

The blood agar plates inoculated from the throat swabs were incubated aerobically in all cases and in some instances also anaerobically.

The specimens from the puerperal and abortional infections were all obtained from cases in this hospital. Two smears and both aerobic and anaerobic cultures were made from either a vaginal or cervical swab, immediately the specimen was taken.

Similarly, in the miscellaneous series of infections smears and aerobic and anaerobic cultures were made with a minimum of delay.

All the cultures were examined after approximately 24 hours' incubation; any haemolytic streptococci present were isolated in pure culture and tested with Lancefield's precipitin sera for Groups A, B, C and G.

Two smears from each specimen were examined. The first was stained with Jensen's modification of Gram and the second with 0·5 p.c. Leishman stain in methyl alcohol.

The Leishman stain was left on the unfixed smear for three minutes, diluted with an equal quantity of tap water or phosphate buffer pH 7 and left on the slide for a further three minutes before washing the slide. In Melbourne tap water has given satisfactory results but where such water contains an appreciable amount of impurities the buffer is preferable. With some batches of Leishman stain satisfactory staining of the streptococcal capsule has been obtained when the stain is let act for one or two minutes instead of three.

# HISTAMINE AND HISTAMINASE IN THE BLOOD OF CASES OF LYMPHOID AND MONOCYTIC LEUKAEMIA

by J. B. THIERSCH[1]

(From the Institute of Medical and Veterinary Science, Adelaide).

*(Accepted for publication 30th September, 1946.)*

Code (1937) failed to detect quantities of histamine in the lymph nodule of the rabbit and horse. Code and MacDonald (1937) found normal blood values ranging from 0·021γ to 0·086γ in cases of lymphoid leukaemia with high lymphocyte counts in the blood varying from 117,000 to 413,000 per c.mm. Went and Kiss (1941) also reported normal histamine blood contents in cases of lymphoid leukaemia. Code (1937) found no increase of histamine in an experimentally produced monocytic pleural exudate of the rabbit.

## EXAMINATIONS.

In the blood of 6 cases of chronic lymphoid leukaemia Code's modification of Barsoum and Gaddum's technique gave the same results as the above-mentioned authors (see Table 1). In no case was more than 0·08γ of histamine per c.c. found in whole blood. The total lymphocyte count varied from 336,000 to 7,000 per c.mm. The platelets were normal in numbers, or scanty.

The histamine and histaminase of the plasma of cases of chronic lymphoid leukaemia were examined (Table 1) as described previously (Thiersch, 1947). In three cases the plasma of untreated patients was investigated. In two further cases with gross enlargement of the spleen and lymph nodules and a high W.B.C. a course of deep X-ray was followed with plasma histamine and histaminase estimations. The plasma was collected in these two cases the day before onset of treatment and from then on after every single exposure to deep X-ray until the full course was completed. The irradiation in both cases consisted of 50r PA and AP to the spleen per dose with a total of 1,000r in the first case and a total of 700r in the second case.

## DISCUSSION.

The low histamine content of the blood of cases of chronic lymphoid and monocytic leukaemias suggests a common origin of the lymphocyte and monocyte.

## SUMMARY.

The blood content of histamine in cases of chronic lymphoid leukaemia in man is normal. This result confirms the work of other workers.

Under deep X-ray treatment no increased amount of histamine or histaminase was found in cases of lymphoid leukaemia.

The histamine content of the blood, spleen and lymph nodules of monocytic leukaemias was found to be normal with Code's method.

The histamine and histaminase content of the plasma of a case of a typical monocytic leukaemia was found to be normal.

*Acknowledgment.* I wish to thank Professor E. R. Trethewie for his help in this work.

## REFERENCES.

Barsoum, G. S. and Gaddum, J. H. (1935): J. Physiol., 81, p. 1.
Code, C. F. (1937): *Ibid.*, 90, p. 485.
Code, C. F. (1937): *Ibid.*, 89, p. 256.
Code, C. F. and MacDonald, A. D. (1937): Lancet, 223, p. 730.
Zon, L. Ceder, E. T. and Crigler, C. W. (1939): Pub. Health Rep., 54, p. 1,978.
Thiersch, J. B. (1947): Austral. J. exp. Biol., 25, p. 73.

[1]Neale Research Pathologist to the Anti-Cancer Campaign Committee, University of Adelaide.

TABLE 1.

*Histamine and histaminase in lymphatic leukaemia.*

| Name. | Date. | Method. | W.B.C. | Total polymorphs. | Histamine in γ per c.c. | Plasma histaminase expressed as γ histamine per ml. after digestion for 5–6 hours. | |
|---|---|---|---|---|---|---|---|
| Br. | 8/8/45 | Direct | 600,000 | — | ·02 | ·12 | 5 hrs. |
| | 21/8/45 | Direct | 330,000 | — | ·08 | ·11 | 5 hrs. |
| | 27/8/45 | Direct | 340,000 | — | Nil | ·12 | 6 hrs. |
| | 29/8/45 | Direct | 340,000 | 3,400 | Nil | ·12 | 5 hrs. |
| post irradiation | | | | | | | |
| 200r | 7/9/45 | Direct | 414,000 | 8,280 | ·04 | ·12 | 6 hrs. |
| 1,000r 200r | 11/9/45 | Direct | 500,000 | — | ·02 | ·14 | 6 hrs. |
| 1,000r 200r | 13/9/45 | Direct | 363,000 | — | ·02 | ·12 | 6 hrs. |
| 1,000r 200r | 23/9/45 | Code | 300,000 | 1,500 | Nil | | |
| 1,000r 200r | 29/9/45 | Code | 340,000 | 3,400 | ·02 | | |
| | 9/10/45 | Direct | 524,000 | — | Nil | ·10 | 6 hrs. |
| | 12/10/45 | Direct | 600,000 | — | — | No histaminase | |
| | 15/10/45 | Direct | 330,000 | — | ·01 | ·10 | 6 hrs. |
| | 17/10/45 | Direct | 299,000 | — | Nil | ·10 | 6 hrs. |
| | 19/10/45 | Direct | 360,000 | — | Nil | ·10 | |
| P.M. | 23/10/45 | Code | Blood | | **·10** | | |
| | | Code | Spleen | | ·35 | | |
| | | Code | Lymph Nodules | | ·26 | | |
| | | Code | Clot | | ·08–·19 | | |
| Fe. | 31/5/45 | Code | 13,300 | 7,200 | ·07 | | |
| no irradiation | 31/8/45 | Direct | 97,000 | 3,870 | ·05 | ·16 | 5½ hrs. |
| | 31/8/45 | Code | 97,000 | 3,870 | ·20 | | |
| | 1/11/45 | Code | 58,000 | 1,000 | ·02 | | |
| Ka. day after irradiation | | | | | | | |
| 100r | 21/8/45 | Direct | 40,000 | — | ·08 | ·06 | 5 hrs. |
| 100r | 23/8/45 | Code | 30,600 | 1,820 | Nil | | |
| | 1/11/45 | Code | 20,900 | 1,000 | ·01 | | |
| Le. | | | | | | | |
| no irradiation | 5/7/45 | Direct | 62,000 | 2,070 | ·07 | ·08 | 5 hrs. |
| | | Code | 62,000 | 2,070 | ·07 | | |
| Pa. | | | | | | | |
| no irradiation | 29/8/45 | Direct | 189,000 | 9,430 | ·04 | ·05 | 5 hrs. |
| | 29/8/45 | Code | 189,000 | 9,430 | ·02 | | |
| 600r 150r | 12/10/45 | Direct | 182,000 | 10,900 | Nil | ·10 | 6 hrs. |
| 600r 150r | 15/10/45 | Direct | 169,000 | 10,900 | Nil | ·10 | 6 hrs. |
| 600r 150r | 17/10/45 | Direct | 62,000 | 3,820 | Nil | ·10 | 6 hrs. |
| 600r 150r | 19/10/45 | Direct | 30,000 | 4,670 | ·05 | ·12 | 6 hrs. |
| 4 days after irrad- | 23/10/45 | Direct | 6,900 | 2,200 | ·01 | ·11 | 6 hrs. |
| 6 days after irrad- | 25/10/45 | Direct | 6,900 | 4,140 | Nil | ·09 | 6 hrs. |
| 10 days after irrad- | 29/10/45 | Direct | 6,300 | 3,150 | Nil | ·12 | 6 hrs. |

On direct examination, with Code's method, of whole blood, spleen and lymph nodules from 3 cases of typical monocytic leukaemia no increased amount of histamine was detected (see Table 2). The plasma histamine was also not increased in one case before and under deep X-ray therapy (see Table 2). The histaminase was found to be slightly higher than normal under deep X-ray therapy, but only a fraction of the amount found in pregnancy.

TABLE 2.

*Histamine in monocytic leukaemia.*

| Name | Date. | Method. | W.B.C. | Total polymorphs. | Total lymphocytes. | Total monocytes. | Histamine in γ per c.c. or per gm. tissue. | Plasma histaminase expressed as γ histamine per ml. after digestion for 5–6 hours. | |
|---|---|---|---|---|---|---|---|---|---|
| Wi. before irradiation | 28/6/45 | Direct | 17,100 | 9,600 | 2,900 | 4,600 | ·03 | nil | 6 hrs. |
| before irradiation | 3/8/45 | Direct | 61,500 | 39,000 | 5,000 | 17,500 | ·09 | 0·10 | 6 hrs. |
| before irradiation | 31/8/45 | Direct | 64,000 | 45,000 | 5,000 | 14,000 | ·02 | 0·11 | 6 hrs. |
| total 125r | 4/9/45 | Direct | 73,000 | 52,000 | 4,500 | 16,000 | ·09 | 0·12 | 6 hrs. |
| total 250r | 7/9/45 | Direct | 71,000 | 49,500 | 4,500 | 17,000 | ·07 | 0·11 | 6 hrs. |
| total 500r | 11/9/45 | Direct | 20,500 | 12,550 | 1,450 | 6,500 | ·01 | 0·11 | 6 hrs. |
| total 750r | 13/9/45 | Direct | 28,300 | 19,500 | 850 | 8,000 | ·01 | 0·10 | 6 hrs. |
| | 31/8/45 | Code | 64,000 | 45,000 | 5,000 | 17,500 | ·06 | | |
| Wi. P.M. | 14/10/45 | | | | | | | | |
| | Blood | Code | 99,000 | 79,000 | 5,000 | 15,000 | ·03 | | |
| | Spleen | Code | No cell counts possible | | | | ·03 | | |
| | Clot | Code | No cell counts possible. | | | | ·03 | | |
| Ti. | 19/9/45 Sternal Puncture | Code | 17,900 | 13,210 | 1,610 | 718 | Nil | | |
| Lo. | 22/5/46 | Direct | 233,000 | 228,000 | 400 | 4,600 | ·07 | | |
| | | Code | 233,000 | 228,000 | 400 | 4,600 | ·04 | | |
| Lo. P.M. | 24/5/46 | | | | | | | | |
| | Blood | Code | 181,000 | 177,000 | 400 | 3,600 | ·10 | | |
| | Lymph Nodule | Code | No cell counts possible. | | | | ·10 | | |
| | Lymph Nodule | Code | | | | | ·04 | | |

# TOXICITIES OF THE OPTICAL ISOMERS OF NICOTINE AND NORNICOTINE

by C. STANTON HICKS AND D. A. SINCLAIR[1]

(From the Department of Human Physiology and Pharmacology, University of Adelaide).

*(Accepted for publication 7th October, 1946.)*

A preliminary test of the toxicity of d-nornicotine to rats was made by Hicks and LeMessurier (1935), who used the partially racemized alkaloid obtained from *Duboisia Hopwoodii*. They found it to be about three times as toxic as l-nicotine. We have made a more exact and more comprehensive study of the toxicities of the optical isomers of both nicotine and nornicotine to rats and guinea-pigs and have also made an attempt to assess the insecticidal powers of d-nornicotine compared with that of l-nicotine, using mosquito larvae as test insects.

## PREPARATION OF COMPOUNDS.

*d-Nornicotine.* The naturally occurring alkaloid is racemized to the extent of about 50 p.c. The dextro form can be separated from the racemic by fractional crystallization of the diperchlorate from methyl alcohol plus ether as described by Späth (1935). It is important to have dry solvents as a small amount of water present leads to the precipitation of an oil instead of crystals on addition of the ether. In such cases the admixture of acetone was found to favour crystallization, but unfortunately, also seems to prevent the separation of the dextro-alkaloid from the racemic. The highest specific rotation obtained was $[\alpha]_D = 84{\cdot}0°$.

*l-Nornicotine.* We obtained a small quantity of l-nornicotine by the method described by Späth (1936), viz., oxidation of l-nicotine with silver oxide. The yield is very small, about 2 p.c. The specific rotation of the product was $[\alpha]_D = -82{\cdot}6°$. A further supply was obtained from the alkaloids of *Nicotiana Excelsior* by fractional extraction.

*l-Nicotine.* Commercial nicotine purified by distillation was used. The specific rotation was $[\alpha]_D = -166°$.

*d-Nicotine.* This we obtained from our d-nornicotine by methylation with formaldehyde plus formic acid (see Späth, 1935). The yield was theoretical and the product of a high degree of optical purity — $[\alpha]_D = 168°$, thus confirming the purity of our d-nornicotine.

## TOXICITY EXPERIMENTS.

*Rats.* The monohydrochlorides of the alkaloids were always used, in solutions made up to have the same total molality as normal saline by the addition of the requisite amount of sodium chloride. This practice was adopted after preliminary experiments had revealed that considerable variation in toxicity could be brought about by varying the concentration of the injected solution whilst keeping the dose constant, due apparently to varying the rate of entry of the drug into the blood stream. Groups of ten animals were injected intraperitoneally with doses ranging about the medium lethal dose and characteristic toxicity curves were constructed by plotting number of deaths against dose expressed as the logarithm of the number of milligrams of alkaloid per kg. of rat. It is better to use the logarithmic scale as this makes the curves the same shape in all regions, whereas plotting against numerical dose gives flatter curves the higher the dose. Trevan (1927) considers that for regular results ten in a group is too small a number, and recommends thirty as being the lowest number that could reasonably be expected to give a smooth curve. However, for our purposes we found the results sufficiently regular and although points did occasionally fall well off the smooth curve, the customary shape of the curve being known, its position could be estimated with reasonable accuracy. For a more refined comparison of two approximately equal poisons, larger number of animals would certainly be necessary. We carried out the experiments on d-nicotine some months later than those on l-nicotine and found a difference of about 15 p.c. between the two curves; but on repeating two

[1]Under grant from the Council for Scientific and Industrial Research.

points with l-nicotine we found that these fell on the d-nicotine curve, indicating that the displacement of the curve was due to a change in the susceptibility of the rats. One series was carried out in winter and the other in summer, and the change in susceptibility was probably due to the temperature or some allied factor.

TABLE 1.

*Number of deaths in each group of ten animals injected with alkaloid dose expressed in milligrams per kg. of animal.*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***Rats.*** | | | | | | | | | |
| d-Nornicotine | Dose (mg./kg.) | 3 | 5 | 6 | 6·5 | 7 | | | |
| | Deaths | 0 | 2 | 4 | 8 | 10 | | | |
| d-Nornicotine (50 p.c. racemic) | Dose | 4·7 | 5·3 | 5·7 | 6·4 | 8·5 | | | |
| | Deaths | 0 | 2 | 4 | 9 | 10 | | | |
| dl-Nornicotine | Dose | 7·5 | 9 | 11 | | | | | |
| | Deaths | 1 | 1 | 5 | | | | | |
| l-Nornicotine | Dose | 15 | 20 | 25 | 29·6 | 35 | 45 | | |
| | Deaths | 0 | 3 | 8 | 7 | 10 | 10 | | |
| l-Nicotine (winter) | Dose | 16·5 | 18 | 20 | 22 | 25 | | | |
| | Deaths | 0 | 1 | 7 | 8 | 10 | | | |
| l-Nicotine (summer) | Dose | 20 | 23 | | | | | | |
| | Deaths | 0 | 4 | | | | | | |
| d-Nicotine (summer | Dose | 20 | 20 | 22·5 | 23 | 25 | 30 | 40 | 50 |
| | Deaths | 2 | 1 | 4 | 5 | 8 | 10 | 9 | 9 |
| ***Guinea-pigs.*** | | | | | | | | | |
| d-Nornicotine | Dose | 8 | 10 | 12 | 15 | | | | |
| | Deaths | 1 | 8 | 9 | 8 | | | | |
| l-Nornicotine | Dose | 17·5 | 30 | | | | | | |
| | Deaths | 1 | 6 | | | | | | |
| l-Nicotine | Dose | 30 | 35 | 35 | | | | | |
| | Deaths | 2 | 9 | 10 | | | | | |
| d-Nicotine | Dose | 30 | 35 | 35 | 40 | 50 | 62·5 | 75 | |
| | Deaths | 1 | 2 | 9 | 9 | 10 | 9 | 9 | |

Our results yield the surprising conclusion that whereas d-nornicotine is 3½ times as toxic as l-nornicotine there is no difference between the two nicotines. One would have expected a similar difference between the pairs of isomers and the fact that there is not, illustrates how complex is the problem of elucidating the mode of action of these drugs. Moreover, unpublished work reveals a marked difference between the nicotine isomers in their action on individual nerve centres such as the cervical ganglion of cats.

Within the limits of experimental error the toxicity of l-nornicotine appears to be identical with that of the nicotines. Also there is no detectable difference in toxicity between the pure dextro and the 50 p.c. racemized nornicotine. The 100 p.c. racemic, however, yields a curve at about the theoretical position between dextro and laevo.

Our findings are in disagreement with the general belief that d-nicotine is only half as toxic as l-nicotine, on the evidence of Mayor (1904) who, however, based his judgment on the results of injections into only one or two animals (guinea-pigs and rabbits), and individual cases can be very misleading. It was possible that there might be a difference between rats and guinea-pigs, which would account for the disagreement. To check this we carried out a similar series of experiments with the latter animals.

TABLE 2.

*Median lethal doses in milligrams per kg. of animal, estimated from curves in Figs 1 and 2.*

| | Rats. | | Guinea-pigs. |
|---|---|---|---|
| Alkaloid. | Season. | M.L.D. | (all in winter). |
| d-Nornicotine | Winter | 6 | 10 |
| l-Nornicotine | Summer | 23½ | 28 |
| dl-Nornicotine | Winter | 10½ | |
| l-Nicotine | Winter | 20 | 32 |
| l-Nicotine | Summer | 23½ | |
| d-Nicotine | Summer | 23½ | 33 |

*Guinea-pigs.* The same technique was adopted as with rats. Owing to the heterogeneity of the stock which was purchased from dealers, the results display somewhat greater irregularity than those with rats. Nevertheless, they are sufficiently clear to show that although these animals are more resistant than rats, the relative positions of the curves are about the same. It is possible that d-nicotine is very slightly less toxic than l-nicotine, but within the limits of experimental error they are the same. Only two points were determined for l-nornicotine which may be slightly superior in toxicity to the nicotines, while d-nornicotine is about 3 times as toxic. Trials with doses of d-nicotine much higher than the median lethal dose showed that a 1 in 10 chance of recovery persisted to as high as twice the median lethal dose. This chance may be due to the chance of the injection gaining access to a larger area of visceral peritoneum.

The chief difference in symptoms noticeable between the drugs was that the nicotines were much more rapid in action than the nornicotines. With a lethal dose of nicotine respiration would cease within ½ to 1 minute after injection followed by violent convulsions, the body being generally stretched out with back straight or concave. Death would ensue in 3 to 10 minutes. In the case of the nornicotines it was some minutes before convulsive fits set in, and these were different in appearance from those of the nicotine-injected animals, consisting of spasmodic jumps, the back remaining convexly arched. Death generally occurred in 6 to 14 minutes.

FIG. 1
CHARACTERISTIC TOXICITY CURVES OF RATS

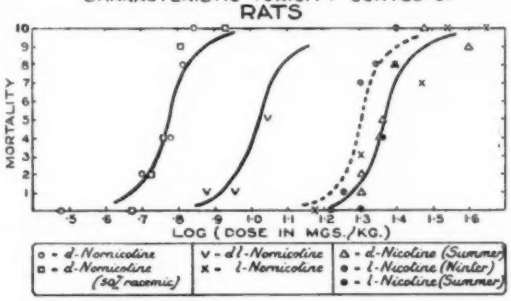
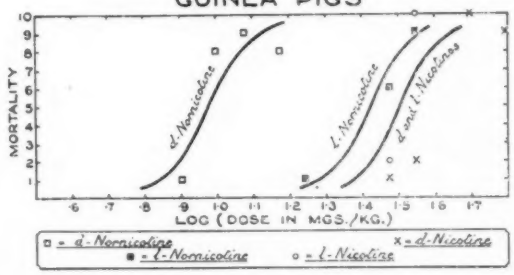



FIG. 2
CHARACTERISTIC TOXICITY CURVES OF GUINEA PIGS




*Mosquito Larvae.*

In view of the widespread use of nicotine as an insecticide, it is of interest to compare the toxicity to insects of nornicotine. Racemic nornicotine has already been tested on *Aphis rumicus* by Richardson, Craig, and Hansberry (1936), who found it to be equal with l-nicotine in toxicity. We made attempts to secure aphides from the Waite Institute, but owing to the depredation of parasites were unable to secure a satisfactory colony. We then turned our attention to mosquito larvae as possible test insects. A salt water species, *Aëdes concolor* Taylor, found in rock pools in New South Wales, seemed suitable, as the adults do not require blood and the larvae were shown by Woodhill (1936) to be capable of adapting themselves to salt concentrations varying from that of Sydney tap-water to several times that of sea water. Adelaide tap-water proved to be unsuitable; but a healthy colony was successfully reared in sea water at 25° C. Preliminary experiments indicated that these salt water larvae were much more resistant to nicotine than the fresh water species experimented with by Campbell, Sullivan and Smith (1933), who found a median lethal concentration of the order of 1 part in 10,000. We found that this concentration had little observable effect and that even with concentrations twenty times this, some still survived next day.

A series of beakers each containing about 50 larvae in 100 c.c. of sea water were treated with the neutral hydrochlorides of d-nicotine and d-nornicotine in amounts ranging from 1 in 250 to 1 in 10,000 of the alkaloids. They were set aside in an incubator at 25° C. until next day. Even in the 1 in 250 solutions some larvae still exhibited tremulous movements, though very feeble in character. Proceeding down the series to the less concentrated solutions, one observed gradually increasing mobility until at 1 in 10,000 no noticeable effects were apparent. In the intermediate solutions some larvae were at the surface with heads up (normally they hang with tails up), while others remained for long periods with heads down at the bottom of the beaker. Numbers of deaths were exceedingly difficult to count, as some larvae remained still for a long time, and though apparently dead, would respond to disturbance with a

needle. Even this was not a satisfactory guide, as some would not respond to slight disturbance but would wriggle on being more violently disturbed, and the dividing line between dead and alive was so vague that the attempt to count deaths was abandoned. In any case the characteristic curves would be so flat that accurate comparison would have been very difficult. General observation indicated that the toxicity of the nornicotine was of the same order as that of nicotine. If anything, it was slightly less toxic but certainly more than half as potent. It was noted that the insects possessed a reflex which caused them to swim against the current and when the water was stirred they tended to remain in the same position. Doubtless this reflex serves to preserve them from being washed out to sea in the natural state. It provided a useful test of the degree of partial paralysis, which begins to be observed in the 1 in 5,000 solution. There were 4 or 5 in the nicotines and 12 in the nornicotine still retaining the reflex. At 1 in 2,000 the reflex is completely gone, but about 10 p.c. of the larvae still seem capable of swimming more or less normally. At 1 in 1,000 no normal swimming is observed—only spasmodic movements and tremors; at 1 in 500 tremors and wriggling more feeble, and at 1 in 250 very feeble and only in one or two cases.

Larvae which had been in the 1 in 250 solutions for 22 hours and others which had been in the 1 in 500 solutions for two days were transferred to fresh sea water. On the following day there were observed about a dozen in each beaker fully recovered. The reason for the amazing resistance of these insects might well form the topic of further research. It is probably associated in some way with their capacity to adapt themselves to wide ranges of salt concentration.

*Acknowledgment.* We are indebted to Mr. D. C. Swan, of the Waite Institute, who reared the mosquito larvae for us.

## SUMMARY.

The relative toxicities of the optical isomers of nicotine and nornicotine to rats and guinea-pigs injected intraperitoneally have been determined, showing threefold difference between d-nornicotine and the others (equal). The larvae of the salt water mosquito *Aëdes concolor* show remarkable resistance to nicotine and nornicotine.

## REFERENCES.

Campbell, F. L., Sullivan, W. N. and Smith, C. R. (1933): J. econ. Entomol., 26, p. 500.
Mayor (1904): Arch. Sci. phys. et nat., 17, p. 418.
Richardson, C. H., Craig, L. C. and Hansberry, T. R. (1936): J. econ. Entomol., 29, p. 850.
Späth, E. and Zajic, E. (1935): Ber., 68, p. 1667.
Späth, E., Marion, L. and Zajic, E. (1936): *Ibid.*, 69, p. 251.
Trevan, J. W. (1927): Proc. Roy. Soc., Biol., p. 483.
Woodhill, A. R. (1936): Bull. entomol. Res., 27, p. 633.